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CXLV. STUDIES IN BACTERIAL NUTRITION.

III. PHOSPHATES AND THE GROWTH OF STREPTOCOCCI.

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(*Received September 11th, 1926.*)

The analytical study of media used for growing bacteria has produced very little chemical knowledge up to the present time. Limiting oneself to the more pathogenic organisms (such as streptococci), the facts so far elucidated may be briefly summarised as follows.

(i) Amino-acids are not so important for growth as was at one time thought. Some amino-acids are favourable to growth (*e.g.* alanine, taurine and asparagine), but many are inhibitory when present in excess [McLeod and Gordon, 1926]. Tryptophan has been definitely excluded as a necessary substance for streptococci [Whitehead, 1924]. Mueller [1922] has isolated a hitherto unknown sulphur-containing amino-acid which he has found to be a growth accessory for streptococci. In general, although a certain amount of amino-nitrogen is always present in good media, the amount of growth is not dependent on the concentration of substances containing this grouping. McLeod and Gordon [1926] give a summary of the work which has been done on this point.

(ii) From another standpoint two substances both essential to streptococcal growth have been demonstrated in a protein medium; one is precipitable by phosphotungstic acid and the other is not [Whitehead, 1923].

(iii) By the use of another method of protein analysis it would appear that some substance concerned in streptococcal growth must be partially soluble in butyl alcohol, but only to a very slight extent. Extraction of a medium for 100 hours by Dakin's butyl alcohol method failed to render it useless, although growth was considerably lessened.

The methods of chemical analysis, which can be applied with any degree of success to a medium consisting mainly of protein derivatives, are very limited, on account of two experimental factors:

(i) the difficulty of avoiding changes in the proteins during manipulation, and of determining whether such changes have taken place;

(ii) the elimination of bactericidal or inhibitory substances, especially excess of neutral salts, when manipulation is finished; otherwise growth experiments on the separated fractions are negative or fallacious.

In any case the most for which we can hope at the present time is to obtain results which will eliminate certain substances as growth accessories and indicate to what class or classes of compounds the active substances belong.

A method so far not investigated in the examination of media is separation of the protein substances by using ethyl alcohol as a precipitant. This agent has the advantage of being readily removable by distillation at a low temperature which seems to render it admirably fitted for the purpose. The present paper describes results obtained by the application of this method.

In the first instance, Cole and Onslow's caseinogen broth, fresh from trypsinisation, was treated with absolute alcohol at 0°, so as to render it first 66 % and then 86 % alcoholic. Two precipitates were obtained and separated by centrifuging. Each was redissolved in water, adjusted to p_H 7·5 and made equal in volume to the original medium. The possibility of change in the protein in these precipitates due to the action of alcohol should not be overlooked, but they were at any rate readily redissolved in water giving clear solutions. The remaining liquid was freed from alcohol by distillation *in vacuo* at 45–50° and similarly made up in water. Thus were obtained three fractions of the original medium:

"A" precipitated by 66 % alcohol.
"B" precipitated by 86 % alcohol.
"C" residual liquid.

Solution C had the characteristic amino-acid smell usually associated with caseinogen broth and contained about 67 % of the amino-N of the original broth. Solutions A and B were odourless and contained about equal amounts of amino-N; A was slightly opalescent.

Growth experiments were performed by distributing the liquids in small plugged tubes, sterilising, and sowing thinly with suspensions of streptococci as described in previous papers. It was found that, whilst none of the fractions singly showed any marked growth, all three together gave growth equal to that in a control tube of untreated broth. A combination of A and C in 24 hours gave almost as much growth as the control tube. A combination of B and C gave no visible growth in 24 hours, but in 48 hours the growth was almost as good as that in AC. That is to say, BC showed a lag period before growth occurred.

By chance a growth experiment was performed in which some disodium hydrogen phosphate solution was added to all the tubes, primarily with the idea of stabilising the p_H. A rather surprising result was obtained. AC now gave no growth in 24 hours and little or none in 48 hours, whilst BC in 24 hours gave growth quite as good as that in the control. A combination of A and B gave a slight growth in the presence or absence of added phosphate. It thus became evident that the phosphate was playing a much more active rôle than that of a mere buffer salt.

On examination it was found that solution A contained 70–80 % of the phosphate originally present in the medium, solution B about 15 % and solu-

tion C about 5 %. The proportion varied, of course, with different batches of medium. Moreover, the phosphate in A appeared to be mainly in the inorganic form, since a granular precipitate of ammonium phosphomolybdate could be readily obtained without previous oxidation of the organic matter. In B and C, on the other hand, treatment with ammonium molybdate without oxidation of the organic matter gave flocculent protein-like precipitates. Estimation of inorganic phosphate in the three solutions confirmed this view: no uncombined phosphate was found in B or C.

At first sight, therefore, it seemed that the above experiments might be explained on the assumption that the organism needed a certain minimum amount of inorganic phosphate or of phosphate in a readily accessible form for its growth, but that a larger amount was inhibitory. Since the phosphate in BC was all in organic combination, the organism could only grow slowly in those solutions until its enzymes had liberated phosphate from combination. So simple a hypothesis was not, however, borne out, or at any rate other factors enter into the problem, for the total concentration of phosphate in BC could be increased to nearly double the value which checked growth in AC without inhibiting the organism in the former mixture. Inhibition in AC was not, therefore, simply an osmotic effect: other substances in solution A must modify the action of the phosphate and a complete explanation of the various phenomena is lacking at present.

Experiments showed that inorganic phosphates were produced when streptococci were allowed to grow in BC for 48 hours, but whether phosphate was liberated for use by the organism, or merely as an incidental happening in its metabolism, is difficult to prove.

The effect of several other salts was tested with some interesting results which should be amplified by more thorough investigation.

Potassium dihydrogen phosphate proved incapable of activating mixture BC, and furthermore no growth occurred in 48 hours. Added to mixture AC it caused no inhibition.

Diammonium hydrogen phosphate activated mixture BC, but not to the same extent as sodium phosphate. It caused no inhibition when added to AC. This salt, however, caused slight growth to occur in the single solutions A, B and C. Its kation NH_4 may enter in some way into the growth of the organisms.

Sodium salts, such as the acetate and sulphate, gave slight growth with BC in 24 hours and good growth in 48 hours. They seemed to shorten the period of lag. No inhibition was caused in AC.

Potassium salts gave similar results to those obtained with potassium phosphate, *i.e.* inhibition in BC and no effect in AC.

It is clear from the foregoing that the nature of both anion and kation of added salts is of importance in the growth of streptococci. Potassium in particular seems to have an adverse influence unless sufficient sodium phosphate is already available in the medium. The main fact is that inorganic phosphate seems to be necessary for the production of growth in 24 hours and phosphate

in some form is probably essential. It is, however, difficult to prepare a protein medium absolutely free from phosphate, so that the latter point cannot be proved at present. Meader and Robinson [1920] maintain that phosphate is essential to the production of haemolysin and observe no effect on growth, but the horse serum which they added to their medium probably supplies the small amount of phosphate necessary for growth.

A point of practical use is that solution A can be disregarded for the present in the study of caseinogen media, since growth equal to that in whole broth is obtained in a mixture of solution B and C + phosphate. This is important as some of the more complex protein derivatives can be removed, and difficulties in experimental manipulation are overcome.

It would appear from the results obtained that three types of compound are concerned in the growth of streptococci on caseinogen broth.

(i) A complex type of protein derivative present in solutions A and B and probably identical with the active substance precipitated by phosphotungstic acid.

(ii) A simpler protein derivative, present in solution C; possibly an amino-acid.

(iii) Phosphates, preferably in inorganic form. Most probably the phosphate plays the part of a co-enzyme in one or more of the reactions by means of which the organism utilises the protein growth factors in its metabolism.

Effect of heating on the phosphate compounds in broth.

During the previous experiments it became evident that only broth which was quite fresh from digestion gave the clear-cut results which have been described. Medium which had been steamed a few times would not give B and C fractions causing a growth lag. At the same time the broth became rapidly darker in colour under the influence of heat and ultimately lost much of its growth-producing power. Also it had been noticed that broth, to which phosphate had been added, darkened and lost its growth power much more rapidly than normal broth, when steamed, so it was decided to investigate the changes which took place on heating, in the hope that light would be thrown on the nature of the protein growth factors.

In the first place the absence of growth lag in solutions B and C prepared from steamed broth was explained by the fact that traces of inorganic phosphate were detected in those solutions. Probably adsorption of phosphate on the flocculent precipitate produced by 66 % alcohol played some part in the removal of inorganic salts from the broth and, as the character of the precipitate was rather different in steamed broth (being much more granular), it seems reasonable to assume that less powerful adsorption allowed some phosphate to remain in the liquid phase.

The three fractions A, B and C, prepared from fresh broth, were steamed for about 12 hours with the addition of all the salts which had been tested

during the growth experiments. Control tubes of whole broth were similarly heated. At the end of this period it was observed that:

(i) A did not darken with or without the addition of any salt whatever;

(ii) B darkened a little without, and a little more with, the addition of phosphate; other salts caused no more darkening than occurred in the control;

(iii) C darkened a little without, and markedly more with, added phosphate; other salts caused no more darkening than occurred in the control;

(iv) tubes of whole broth darkened considerably with no addition and still more with the addition of phosphate; other salts again had no effect.

The tubes of whole broth were sown, after heating, with a streptococcus and while the broth heated alone showed some diminution of growth in the tube containing added sodium phosphate, growth was completely inhibited and greatly delayed in the tubes containing potassium and ammonium phosphates.

The broth which had been heated with additional sodium phosphate proved to have no inhibitory effect on fresh medium. It appeared, therefore, that phosphate had combined with, or changed, some growth substance present mainly in the solution C, thereby rendering it inactive as a growth factor.

The next point was to determine whether phosphate had combined with the growth factor, for this might afford valuable clues as to the nature of the protein derivative. Inorganic and organic phosphate were estimated in fresh and in heated broth, and it was found that in every instance there was an increase in inorganic phosphate after heating. When the heated broth was separated by 66 % alcohol, an increased amount of phosphate was found in the portion corresponding to fractions B and C but the increase was accounted for by inorganic phosphate, the concentration of combined phosphate being practically the same as in fresh broth. In effect there was no evidence whatever that inorganic phosphate had recombined with protein substances under the influence of heat. On the contrary, the results showed that the main change in phosphate distribution on heating was a hydrolysis of some of the organic phosphorus compounds in fraction A.

The change of the growth substance present in C must therefore be one which is catalysed by phosphates. Experiments in which broth was heated in narrow-necked flasks well stoppered, and in Erlenmeyer flasks giving a large liquid surface, showed that the reaction was probably not an oxidation, since the darkening was absolutely equal under both conditions. It can only be said, therefore, that one of the growth factors for the streptococcus, one of the simpler protein derivatives, is changed on heating in the presence of phosphates, so that it no longer supports growth of the organism.

Experimental.

Separation of broth by alcohol.

25 cc. of fresh caseinogen broth were poured into 50 cc. of ice-cold absolute alcohol and the mixture allowed to stand for 1 hour in the ice-chest. The flocculent white precipitate (A) was separated by centrifuging and the supernatant fluid poured into 100 cc. of cold alcohol. After another hour the second precipitate (B) was separated by centrifuging. Both precipitates were washed with the appropriate strength of alcohol and then dissolved in water to the original volume of broth, and adjusted to p_H 7·5. The alcoholic liquid (C) remaining from the separation was evaporated to dryness *in vacuo* at 45–50°, redissolved and adjusted. The following table gives the growth results obtained with a haemolytic streptococcus. Experiments with two other strains bore out the results.

Table I.

Diluent	A	B	C	AB	AC	BC	ABC	Control
Water	Trace	–	Trace	+	+ + +	Trace (+ + +)	+ + + +	+ + + +
Na_2HPO_4	„	–	+	Trace	–	+ + + +	.	+ + + (+ + + +)
KH_2PO_4	„	–	–	„	+ + +	–	.	+ + + +
$(NH_4)_2HPO_4$	„	Trace	Trace	+	+ + +	+ + +	.	+ + + +
Na_2SO_4	„	–	„	+	+ + +	+ (+ + +)	.	+ + + +
K_2SO_4	„	–	„	+	+ + +	–	.	+ + + +

The results in brackets indicate changes which took place in 48 hours. Trace indicates no visible growth, but growth obtained on subculturing.

+, + +, + + +, + + + + are degrees of growth estimated by opacity.

The salts used were all adjusted to p_H 7·5 and had an effective concentration of $M/45$ in the growth tubes. Dilutions were arranged so that solutions A, B and C and the broth control were one-third of their original strength in every tube.

Phosphate estimations.

Inorganic phosphate was estimated in the solutions by precipitation with magnesia mixture. Total phosphate was estimated by oxidation and precipitation as ammonium phosphomolybdate, the latter being estimated volumetrically.

Inorganic and organic phosphate in solutions A, B and C separated from fresh broth.

	A		B		C	
Exp.	Inorganic	Organic	Inorganic	Organic	Inorganic	Organic
1	0·147	0·097	0·0	0·029	0·0	0·018
2	0·191	0·106	0·0	0·049	0·0	0·019
3	0·129	—	0·0	—	0·0	—
4	0·127	0·049	0·0	0·060	0·0	0·033

Results expressed as % P_2O_5.

Change in distribution of phosphate on heating broth.

I. Three samples of broth were prepared and treated as follows.

(i) Steamed 10 minutes and adjusted to p_H 7·5.

(ii) Steamed for 12 hours in 100 cc. graduated flask with narrow neck, tightly stoppered.

(iii) Steamed 12 hours in an Erlenmeyer flask with large liquid surface.

Samples (ii) and (iii) were subsequently adjusted in reaction (they were very slightly acid) and brought to their original volume with distilled water. When placed in similar flasks it was observed that both heated samples had darkened to the same extent, so that probably oxidation had nothing to do with the phenomenon of darkening.

Phosphate concentration (% P_2O_5).

(i)		(ii)		(iii)	
Inorganic	Organic	Inorganic	Organic	Inorganic	Organic
0·106	0·104	0·133	0·075	0·135	0·076
Totals 0·210		0·208		0·211	

Heating therefore causes a decomposition of organic phosphorus compounds and liberation of additional inorganic phosphate.

II. Samples of fresh broth and broth steamed for varying times were treated with 66 % alcohol at 0°. After centrifuging, the distribution of phosphate was investigated in the supernatant fluid (corresponding to solutions B and C). The reaction of all the samples was p_H 7·5 at the time of separation.

Time of heating:	¼ hour		2 hours		6 hours		12 hours	
	Inorganic	Organic	Inorganic	Organic	Inorganic	Organic	Inorganic	Organic
% P_2O_5	0·000	0·093	0·007	0·087	0·013	0·098	0·031	0·073
	*0·003	0·089	0·007	0·087	0·017	0·082	0·037	0·062

* Solutions B and C prepared from this particular sample of broth showed a growth lag of less than 24 hours. This is accounted for by the trace of inorganic phosphate.

No great accuracy is claimed for the figures giving inorganic phosphate in the supernatant fluid, since the amounts were very small, but the results give quite definite evidence that the darkening caused by heat is not accompanied by a combination of phosphate with the substances in the solutions B and C. At the same time it is evident why B and C show no growth lag when prepared from heated broth, since inorganic phosphate is present in those solutions and not in those prepared from fresh broth.

Concentration of amino-N and total N in the three fractions of broth.

The following table gives concentrations of amino- and total N found in a typical experiment.

	A	B	C
% Amino-N	0·092	0·092	0·378
% Total N	0·224	0·227	0·648

SUMMARY.

1. A tryptic digest of caseinogen may be separated into three fractions by treatment with ethyl alcohol. All the inorganic phosphate is present in the fraction precipitated by 66 % alcohol.

2. Streptococci have a lag period of about 24 hours before growth occurs in a medium made from the two fractions containing no inorganic phosphates. Rapid growth occurs if phosphate is added to these fractions.

3. From the results obtained at least three factors appear to be concerned in the growth of streptococci in caseinogen medium; two are probably protein derivatives and the third is phosphate preferably in inorganic form.

4. The darkening and deterioration of caseinogen medium which occur on steaming are probably due to the presence of phosphate.

5. The deterioration is due to a growth factor, present among the simpler protein derivatives, being rendered inert. The change is not an oxidation nor is there a combination of the growth factor with phosphate, but phosphate appears to catalyse the reaction.

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CXLVI. A THERMAL STUDY OF A POSSIBLE SOURCE OF ENERGY IN ANAEROBIC METABOLISM WITH SOME NOTES ON THE PROPERTIES OF SORBITOL.

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(*Received September 28th, 1926.*)

Introduction.

In order to study the energy-producing mechanism in the living cell under anaerobic conditions, it is first necessary to ascertain, as far as possible, what reactions take place. The only one which has so far been established is the glycolytic formation of lactic acid, although several others have been suggested as the possible source of energy. The evidence for the existence of these reactions in the living organism is being examined in each case, in order to determine whether any amongst them may be of importance in anaerobiosis. The production of valeric acid by *Ascaris lumbricoides* has been dealt with by one of the authors [Slater, 1925] in a previous communication, and the present work deals with the second reaction investigated.

Amongst the sources of anaerobic energy which have been suggested, the auto-oxidation and reduction of dextrose after the manner of the Cannizzaro reaction [Mathews, 1921] is perhaps the best supported by collateral evidence; but, unfortunately, no direct proof is available for the existence of such a process in the animal body.

It has long been known that the small quantities of fatty aldehydes existing in the animal organism can be converted into the corresponding acids. Further, it has been shown that isolated tissues have the power to bring about this reaction. It was at first assumed that the only factors involved were the aldehyde and the free oxygen reacting under the influence of aldehydease [Dakin, 1922].

The work of Dony-Hénault and Van Duuren [1907], amongst others, showed that free oxygen is not essential to the reaction, and that in certain circumstances its presence actually retards the formation of the acid. Parnas [1910] and Battelli and Stern [1910] were able to prove that the formation of the

fatty acid is not a simple oxidation, but a hydrolytic auto-oxidation and reduction of the same type as benzaldehyde undergoes when shaken with strong aqueous potash. This change may be represented by the equation:

$$2R.CHO + H_2O = R.COOH + R.CH_2OH.$$

The last named workers further demonstrated that the reaction was dependent on an enzyme "aldehydemutase," which appeared to occur largely in the liver. Parnas [1910], calculating from the known heats of combustion of the materials involved, showed that the reaction is, in the case of the fatty aldehydes considered, exothermic, and hence a potential source of energy.

Owing to the highly reactive character of dextrose in the presence of small quantities of oxygen, it has not yet been possible to show whether the aldehyde group of the molecule acts in this way or not. The difficulty is increased by the possible changes which may take place in the end-products of the reaction—gluconic acid and *d*-sorbitol.

If, however, it can be demonstrated that the reaction in the case of dextrose is exothermic, additional weight will be lent to the evidence already available in support of the hypothesis; whilst if, on the other hand, the reaction should prove to be endothermic, further investigation on the subject can be abandoned.

In order to calculate the heat exchanges in the reaction it is necessary to know the heat of combustion of gluconic acid, dextrose and *d*-sorbitol in solution. The value for this constant has been determined in each case, the actual figures being:

Dextrose	676·08 Cals.	per g. mol.
Gluconic acid	618·59 ,,	,,
Sorbitol	732·44 ,,	,,

The energy change is therefore equal to

$$\frac{(2 \times 676{\cdot}08) - (618{\cdot}59 + 732{\cdot}44)}{2}$$

or 0·57 Cal. per g. mol.

It is immediately obvious that although such a reaction would be exothermic, the heat available is so small that compared with the breakdown of glycogen to lactic acid it is negligible.

There appear, therefore, to be good grounds for rejecting the suggestion that under anaerobic conditions dextrose reacts in this way, with the production of the necessary energy for the maintenance of life.

Determination of the heat of combustion of dextrose.

The heat of combustion of dextrose has been previously determined by Stohmann and Langbein [1892], who obtained the figure 3743 g. cals. per g., and by Amery and Benedict [1911], whose value was 3739 g. cals. per g. Although these values were in excellent agreement, some doubt remained as to the state of hydration of the dextrose used. The heat of combustion was therefore determined on a carefully purified sample of dextrose, and an average

value of 3739 g. cals. per g. was obtained. As the value is in agreement with the previous determinations, the details of the work are given as briefly as possible.

The purification of the dextrose and its combustion.

A sample of commercially pure dextrose was crystallised several times by Soxhlet's method from methyl alcohol and water, and after preliminary drying in air was heated at 80° until a constant weight was reached.

The material so obtained was burnt in the Mahler-Cook bomb used by one of the authors for the determination of the heat of combustion of glycogen [Slater, 1924], the same calibration figure being employed. Collodion was used to fire the dextrose, and this is allowed for in the calculations. Four determinations are given below.

Heat equivalent of bomb = 760·5 g. cals.
Heat of combustion of collodion taken as 2780 g. cals. per g.

	Weight of glucose (g.)	Weight of collodion (g.)	Weight of water in calorimeter (g.)	Rise in temperature (° C.)	Heat of combustion (g. cals. per g.)
(1)	0·9994	0·0356	2489·7	1·178	3734·1
(2)	1·0039	0·0704	2488·8	1·218	3746·5
(3)	1·0004	0·0197	2485·2	1·167	3731·5
(4)	1·0371	0·0263	2481·5	1·221	3743·9

Average value = 3739·0 g. cals. per g.

Heat of solution of dextrose.

The method used for the determination was the same as that for glycogen [Slater, 1924]. The water equivalent of the flask found by electrical calibration was 16·9 g. cals. per ° C. The following table gives the results of five determinations.

	Weight of dextrose (g.)	Total heat equivalent (g. cals.)	Change in temperature (° C.)	Heat of solution (g. cals. per g.)
(1)	1·0030	166·9	−0·105	−17·5
(2)	1·0001	168·7	−0·095	−16·0
(3)	1·0018	167·6	−0·099	−16·6
(4)	1·0015	167·2	−0·101	−16·9
(5)	0·9992	169·3	−0·112	−18·7

Average value = −17·1 g. cals. per g.

From these results the heat available from 1 g. of dextrose in solution is 3756·1 g. cals., or 676·08 Cals. per g. mol.

DETERMINATION OF THE HEAT OF COMBUSTION OF GLUCONIC ACID.

Gluconic acid has not been obtained in a pure state, the best preparation consisting of a syrup of unknown water content. An indirect method had therefore to be adopted. After some preliminary experiments and a general consideration of the possible methods, it was decided to use the following four reactions:

(1) $C_6H_{12}O_7(aq) + \frac{Ca}{2}(OH)\,aq = \frac{Ca}{2}\,OOC.C_5H_{11}O_5 . \frac{1}{2}H_2O(aq) + A$ Cals.
gluconic acid

(2) $\frac{Ca}{2}\,OOC.C_5H_{11}O_5.\frac{1}{2}H_2O\,(aq)=\frac{Ca}{2}\,OOC.C_5H_{11}O_5.\frac{1}{2}H_2O+aq+B$ Cals.

(3) $\frac{Ca}{2}\,OOC.C_5H_{11}O_5.\frac{1}{2}H_2O+\frac{11}{2}\,O_2=\frac{Ca}{2}\,(OH)+6CO_2+\frac{11}{2}\,H_2O+C$ Cals.

(4) $\frac{Ca}{2}\,(OH)+aq=\frac{Ca}{2}\,(OH)\,aq+D$ Cals.

By summing these equations we get

(5) $C_6H_{12}O_7\,(aq)+\frac{11}{2}\,O_2=6CO_2+\frac{11}{2}\,H_2O+(A+B+C+D)$ Cals.
gluconic acid

Preparation of the calcium gluconate.

A specimen of commercially pure calcium gluconate (Kahlbaum) was purified after the manner described by Blanchetière [1923], and finally carefully dried to constant weight at 50°. This sample was shown to lose weight equivalent to 1 molecule of water for each molecule of salt on drying to constant weight at 120°, and to contain the correct amount of calcium.

% loss on drying	Found 4·04	Theory 4·02
% calcium	Found 8·85	Theory 8·92

Determination of the heat of combustion of calcium gluconate.

The production of calcium hydroxide in the combustion provided a difficulty as the carbon dioxide formed combined with it to a limited extent forming calcium carbonate. It was found, however, that, under constant conditions as to the weight of material used and as to the pressure of oxygen in the bomb, the percentage of calcium hydroxide converted was relatively constant. Thus determinations made by titrating the carbonate present gave values between 16 % and 18 %. The heat exchanges in the conversion of the hydroxide to carbonate are known and are given by the equation

$$Ca(OH)_2 + CO_2 = CaCO_3 + H_2O + 29{\cdot}6 \text{ Cals.}$$

The weight of calcium gluconate taken in each combustion was approximately one gram, yielding 0·165 g. of hydroxide. If 17 % of this material is converted into carbonate, the heat evolved will be 11 g. cals. It is obvious, therefore, that small variations in the amount of carbonate found will not materially affect the result, and 11 g. cals. is therefore applied as a correction to the average result of the separate determinations. The combustions were made exactly as in the case of dextrose.

Heat equivalent of the bomb = 760·5 g. cals.
Heat of combustion of collodion taken as 2780 g. cals. per g.

	Weight of calcium gluconate (g.)	Weight of collodion (g.)	Weight of water in calorimeter (g.)	Rise in temperature (° C.)	Heat of combustion (g. cals. per g.)
(1)	1·0009	0·0327	2477·2	0·839	2626·1
(2)	1·0005	0·0255	2479·2	0·830	2616·7
(3)	1·0003	0·0220	2493·1	0·823	2615·7
(4)	1·0042	0·0198	2491·0	0·829	2629·2
(5)	1·0007	0·0248	2501·1	0·827	2626·5
(6)	1·0019	0·0366	2501·0	0·835	2616·5

The average of these figures is 2621·8, from which must be subtracted the 11 g. cals. due to the formation of carbonate, giving the final value for the heat of combustion as 2611 g. cals. per g. or 584·86 Cals. as the value of C in equation (3).

Determination of the heat of neutralisation of gluconic acid.

Solutions of calcium hydroxide and gluconic acid were prepared, the former by shaking pure calcium oxide with water free from carbon dioxide and filtering the undissolved material, and the latter by decomposing a solution of calcium gluconate quantitatively with oxalic acid. The solutions so obtained were adjusted to equivalent strengths, the calcium hydroxide solution containing 1·2861 g. per litre, and the gluconic acid 6·808 g. per litre.

100 cc. of the calcium hydroxide solution were placed in a flask with a Bechmann thermometer and allowed to come to thermal equilibrium in a constant temperature bath. In a vacuum flask in the same bath 100 cc. of the gluconic acid solution were adjusted to the same temperature, and the solutions rapidly mixed. The rise in temperature was noted and corrections for cooling made. The heat equivalent of the vacuum flask, stirrer and thermometer had previously been determined by electrical calibration.

Although this method for determining the heat of neutralisation is open to some criticism, it gave uniform results, and in the opinion of the authors is sufficiently accurate for the present purpose, the maximum deviation from the mean being less than 2 g. cals. per g. The results obtained are given in the table below. In each experiment 0·6808 g. of gluconic acid was neutralised by 0·1286 g. of calcium hydroxide. The total heat equivalent of the calorimeter and its contents was in each experiment 213·4 g. cals.

	Rise in temperature (° C.)	Heat of neutralisation per g. of gluconic acid (g. cals.)
(1)	0·133	41·69
(2)	0·140	43·88
(3)	0·129	40·44
(4)	0·138	43·26
(5)	0·130	40·75

This gives a mean value of 42 g. cals. per g. of gluconic acid, or a value for A in equation (1) of 8·23 Cals.

Determination of the heat of solution of calcium gluconate.

The determination of the heat of solution of calcium gluconate proved a somewhat difficult operation. The low solubility and the slow rate of solution tended to introduce considerable errors. Ultimately it was found most satisfactory to use only small quantities of salt, and to carry out the whole operation at 40°. Otherwise the technique was as described for dextrose. The results obtained in this way were fairly concordant, but were not so uniform as the others under consideration in the present investigation. The extent of the

variation, however, was not sufficiently large to affect materially the final result. The figures obtained are given in the following table.

	Weight of calcium gluconate (g.)	Fall in temperature (corrected) (° C.)	Total heat equivalent of calorimeter (g. cals.)	Heat of solution of calcium gluconate (g. cals.)
(1)	0·0500	0·040	133·6	− 106·9
(2)	0·0502	0·039	133·6	− 103·8
(3)	0·0501	0·041	133·4	− 109·2
(4)	0·0488	0·041	134·2	− 112·7
(5)	0·0450	0·035	133·7	− 104·0

This gives an average value of − 107·6 g. cals. per g., and for B in equation (2) a value of 24·10 Cals.

Heat of solution of calcium hydroxide.

This value has been determined by Thomsen [1882] to be 2·79 Cals. per g. mol.; hence D in equation (4) is equal to 1·395 Cals.

Heat of combustion of gluconic acid in dilute solution.

The sum of the heat exchanges in equations (1), (2), (3) and (4) gives a total value of 618·59 Cals. per g.mol., or 3151 g. cals. per g. for the heat available when gluconic acid in solution is oxidised completely to carbon dioxide and water.

Heat of combustion of *d*-sorbitol.

Preparation and purification of sorbitol.

The literature dealing with the chemical and physical properties of sorbitol is indefinite and lacking in concordance. A general examination of the properties of sorbitol was therefore made, and its synthetic preparation attempted, before a sample was finally selected for combustion purposes. The factors of greatest importance in the present work are the ash and moisture content.

Properties of sorbitol and some of its derivatives.

Moisture content. Sorbitol unlike mannitol does not crystallise readily from water or alcohol-water mixtures, and when crystals are obtained they do not appear to have a definite water content. The information in the literature is as follows. Vincent and Delachanal [1889] obtained needles of indefinite composition from 95 % alcohol. Boussingault [1872] and Hitzermann and Tollens [1889], working with sorbitol from natural sources, isolated a monohydrate melting between 55° and 60°. Fischer [1890], using synthetic sorbitol, obtained a half hydrate by drying over sulphuric acid at room temperature for 3 days; melting-point 75°. Nanji and Paton [1924] obtained what appeared to be anhydrous sorbitol by precipitation with absolute alcohol. This material was very hygroscopic.

General behaviour of sorbitol exposed to air and dehydrating agents. The sample used in the following experiments was purchased from Kahlbaum, and

appeared to be of good quality, except in respect of moisture content. A portion of this was dried at 110° over phosphorus pentoxide *in vacuo*, using the apparatus of Harden and Young [1902]. The loss at constant weight was 3·4 %, as compared with the half hydrate 4·5 %, and the monohydrate 9·0 %. The anhydrous material so obtained was very hygroscopic, and it was therefore concluded that the original sample was a mixture of hydrates. On drying over sulphuric acid at room temperature, the loss at constant weight after 10 days was almost identical, viz. 3·5 %.

On exposure to air, the sorbitol lost or gained weight according to the moisture content of the air. The maximum water content absorbed was 33 %, the sorbitol dissolving at approximately 11 %, and recrystallising when the moisture content fell below this value.

It was concluded that sorbitol forms a series of hydrates which are stable over only small ranges of vapour pressure, and consequently that it cannot be isolated in a stable form.

In order to show that the material obtained on drying was anhydrous sorbitol, samples were taken simultaneously for drying and ultimate analysis. The following results were obtained:

	%
Loss on drying	4·36
Carbon found	38·18
Carbon required if the residue is anhydrous sorbitol ...	37·83
" " " " anhydride (less $\frac{1}{2}H_2O$)	39·80
" " " " hydrate (plus $\frac{1}{2}H_2O$)	36·02

Although the agreement is not good owing to the nature of the experiment, it appeared to warrant the assumption that anhydrous sorbitol results on drying.

Methods of identification and purification. The variations in the melting-point found in the literature were confirmed, it being early evident that unless the moisture content could be stabilised, melting-point determinations were of no value. The optical rotation was also examined as a possible test of purity, but this constant is too small to be of use.

It was therefore decided to prepare some of the derivatives of sorbitol which might serve as a means of identification, and possibly of purification. The dibenzal, which was first prepared by Meunier [1891], was made and examined. The method used was that of Meunier with some slight modifications in the extraction. A mixture is made of a thick aqueous syrup of sorbitol, 50 % sulphuric acid and benzaldehyde in the ratio 1 : 1 : 0·8, from which the dibenzal separates as a paste on standing. The reaction mixture is diluted with water and the paste filtered off and boiled with a dilute solution of sodium carbonate. The addition of the carbonate prevented the decomposition of the dibenzal by traces of acid which cannot be removed by washing with water on the pump. The solid is filtered off when the smell of benzaldehyde is no longer detectable, and washed with water. If it is coloured it is advisable to wash with a little alcohol, and then with ether. The dibenzal is dried in an oven

at 80°, higher temperatures causing decomposition, and should then be a white odourless amorphous powder. Attempts to find a solvent suitable for recrystallisation proved abortive. The melting-point is markedly affected by small traces of benzaldehyde, and by the slow decomposition below the melting-point. The highest value obtained was 179–184°. Meunier claims to have isolated two forms, the one melting at 163° and the other at 200°. It has been found impossible to prepare the second form, and it appears probable that it may have been impure tribenzal mannitol which melts at 213–217°.

Hexa-acetylsorbitol was prepared exactly as described by Tutin [1925], and specimens obtained both from natural and synthetic sorbitol melted sharply at 99° after recrystallisation from ethyl acetate. This derivative proved to be by far the most satisfactory material both for the identification and the purification of sorbitol.

It is obviously a matter of great difficulty to show the presence of small quantities of other hexitols in a specimen of sorbitol, but fortunately for the present work the effect of such impurities on the heat of combustion would be exceedingly small, as they themselves have a heat of combustion approximately the same as sorbitol.

The dibenzal can be obtained in the best yields and was used as a rough assay of the sorbitol present in any product under investigation.

For purposes of purification it was found best to prepare first the dibenzal, which was then decomposed by dilute sulphuric acid in the presence of a little benzaldehyde; the acid was removed by baryta and the benzaldehyde by steam distillation. The product was a syrup, which was free from impurities other than the hexitols. To remove these the hexa-acetyl compound was prepared and repeatedly recrystallised from ethyl acetate. When a product with a sharp melting-point had been obtained, it was decomposed by the method described by Tutin [1925]. Unfortunately, this lengthy process resulted in very poor yields, and was of value only for the preparation of small quantities of material of high purity.

Reduction of dextrose.

The reduction of dextrose by metallic reducing agents has been carried out by numerous workers, with varying degrees of success. It is unfortunate that in many cases the yields are not given, and also, owing to the difficulty of identifying sorbitol, the state of purity of the final product is often in doubt.

The method most generally known is that of Fischer [1890] in which the reduction is carried out with sodium amalgam, the solution being frequently neutralised by the addition of sulphuric acid, so that it is first faintly acid and then faintly alkaline. Meunier [1891], on the other hand, uses a concentrated solution of dextrose and sodium amalgam, the reaction mixture being allowed to become strongly alkaline. In neither case is the yield of sorbitol given.

Neuberg and Marx [1907] carried out the reduction by means of metallic calcium, the solution being kept nearly neutral by means of a stream of carbon

dioxide. No definite yield is given. More recently Nanji and Paton [1924] have used aluminium amalgam for the reduction, the solution being rendered alkaline by ammonia. They claim to have obtained a 60 % yield, but give no data as to the purity.

On considering these methods it was decided to try first that of Fischer, as in the other cases the dextrose was reduced in a definitely alkaline medium, with the obvious possibility of the formation of the mixture of the substances known to be yielded by dextrose in such a solution, together with the reduction products of these substances.

It is necessary to point out, at this stage, that the disappearance of the reducing power of the dextrose cannot be taken as a measure of the sorbitol formed. The only satisfactory method is to weigh the sorbitol as the dibenzal. Although this may not give the total sorbitol formed, it does give the more important value, the amount of sorbitol which can be extracted.

The method described by Fischer was carefully repeated, the solution being frequently neutralised. After several hours' treatment with amalgam no reduction had taken place. The experiment was repeated with exactly similar results. It was found, however, that if the neutralisation was less frequent, the dextrose began to disappear. This might have been due to the production of sorbitol or to the alkaline decomposition of the dextrose.

In order to test this a reduction was attempted in which the solution was maintained throughout alkaline in reaction, acid being added from time to time in quantities sufficient to bring the solution back to p_H 8–9. The solution consisted of 45 g. of dextrose in a litre of water, the sodium amalgam ($2\frac{1}{2}$ %) being added in quantities of 100 g. as it was used up. The experiment was carried out at room temperatures and with only occasional shaking. When 1000 g. of amalgam had been added over a period of 3 days, the reducing power of the solution had fallen to 10 %. After the solution had been evaporated *in vacuo* and extracted with absolute alcohol, a yield of dibenzal was obtained corresponding to 30 % formation of sorbitol.

The by-products were examined and it was found that mannitol had been formed in considerable quantities (5–10 %), and a certain amount of syrupy and gummy material, which appeared to be a mixture containing some sorbitol, mannitol and unchanged dextrose. Meunier's method was also tried, but the by-products formed in this case rendered the separation of the dibenzal difficult, and the yield was consequently low. The method of Neuberg and Marx proved unsatisfactory owing to the large bulk of carbonate precipitated, from which the whole of the sorbitol is only removed after prolonged washing. This difficulty in washing away sorbitol was found to apply to other bulky precipitates.

Reduction with aluminium amalgam as recommended by Nanji and Paton was only moderately successful, yields of not more than 10 % being obtained when the sorbitol was extracted and weighed as dibenzal. It is interesting to note in this connection, that the ammonia which Nanji and Paton believe to

play an important part in the reduction, may be replaced with equally good results by a sufficient quantity of caustic soda to reproduce a similar hydrogen ion concentration. It should be stated, however, that the aluminium used in the present experiment may not have been so good as that used by Nanji and Paton, as it appeared to react more slowly. Three different samples of aluminium were tried, but none of them proved entirely satisfactory.

The best yield obtained was that from the modification of Fischer's method. It is probable that Fischer actually allowed his reaction mixture to become more strongly alkaline than he states, and in this way obtained reduction.

It is unfortunate that the conditions essential to the reduction of dextrose are also those which produce a large number of by-products and an impure end-product. In the present work, where it is essential that pure material should be combusted, sorbitol prepared by reduction is useless, unless it has first been converted into the hexa-acetyl derivative and carefully purified by repeated recrystallisation from ethyl acetate.

The combustion of sorbitol.

After it had been decided that the reduction of dextrose would not yield sorbitol of sufficient purity for the combustion in the necessary quantities, the question of its extraction from natural sources, or its purchase, was considered. Owing to the seasonal difficulties and the low yields obtained either from mountain ash berries or from apples, it was found better and cheaper to use the commercial product.

A very pure sample was fortunately obtained, which was ash-free and showed no reducing power. As far as could be ascertained it had been extracted from natural sources. When a portion of the material was converted into the hexa-acetyl derivative, the product from the ethereal solution after once recrystallising from ethyl acetate melted sharply at 98°. It was concluded from this, that the original material did not contain any notable impurity capable of forming an acetyl derivative under the conditions of acetylation employed.

In order to overcome the difficulty of the irregular moisture content a quantity of about 15 g. was thoroughly powdered and mixed, and placed in a well stoppered bottle. The moisture content was determined by drying and the material used for the combustion was put in the crucible as quickly as possible and weighed in a stoppered tube. The combustion was carried out as in the case of dextrose and calcium gluconate, it being necessary, however, to use a larger quantity of cotton to fire the sorbitol. In order to obtain the accurate correction for the cotton used, its heat of combustion was determined.

Heat of combustion of cotton used for ignition.

Heat equivalent of the bomb = 760·5 g. cals.

	Weight of cotton (g.)	Water in calorimeter (g.)	Rise in temperature (° C. corrected)	Heat of combustion (g. cals./g.)
(1)	0·6317	2504	0·750	3875·3
(2)	0·8030	2501·5	0·955	3878·7

Mean value = 3877 g. cals. per g.

Heat of combustion of sorbitol.

Heat equivalent of the bomb = 760·5 g. cals.
Correction for the moisture content of the sorbitol = 4·36 %.

	Weight of sorbitol (uncorrected) (g.)	Weight of water in calorimeter (g.)	Rise in temperature (corrected) (° C.)	Weight of cotton (g.)	Heat of combustion (g. cals./g.)
(1)	0·5840	2500	0·716	0·0266	3995·3
(2)	0·5981	2503	0·726	0·0200	4003·7
(3)	0·6366	2500	0·790	0·0366	3997·4
(4)	0·7120	2504	0·867	0·0295	3988·5
(5)	0·7957	2502	0·976	0·0330	4016·1
(6)	0·6426	2501	0·812	0·0485	4003·3

Mean value = 4000·7 g. cals. per g.

Heat of solution of sorbitol.

The determination was made in the manner previously described, precautions being taken to prevent the sorbitol absorbing moisture before it was dropped into the water. The same sample of sorbitol was used as that from which the material for the heat of combustion had been drawn.

	Weight of sorbitol (uncorrected) (g.)	Fall in temperature (° C.)	Equivalent of calorimeter + water (g.)	Heat of solution (g. cals./g.)
(1)	0·2498	0·052	115	−24·9
(2)	0·1829	0·040	114	−26
(3)	0·3779	0·073	115	−22·8

Moisture content of sorbitol = 4·36 %.
Mean value = 24·5 g. cals. per g. of anhydrous sorbitol present in the moist sample.

Heat of combustion of sorbitol in solution.

From the two determinations we have the heat of combustion of sorbitol in dilute solution equal to 4025 g. cals. per g. of anhydrous sorbitol dissolved, or 732·44 Cals. per g. mol.

SUMMARY.

(1) The heats of combustion of dextrose, gluconic acid and sorbitol in dilute solution have been determined.

(2) It has been shown that the auto-oxidation and reduction of dextrose to gluconic acid and sorbitol produces only a small amount of heat, and hence can be neglected as a source of anaerobic energy.

(3) The chemical and physical properties of sorbitol and two of its derivatives have been examined.

(4) The conditions for the reduction of dextrose to sorbitol by metallic reducing agents have been investigated.

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CXLVII. THE AEROBIC AND ANAEROBIC METABOLISM OF THE COMMON COCKROACH (*PERIPLANETA ORIENTALIS*). PART I.

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Introduction.

When the common cockroach (*Periplaneta orientalis*) is deprived of oxygen, it enters into a state closely resembling anaesthesia. This condition can be produced by replacing the air by nitrogen or any inert gas, by immersing the cockroach in boiled water, or even by evacuating the vessel in which it is contained.

If the deprivation has not been continued for too long a time, the readmission of air quickly revives the cockroach, and after a time depending upon the duration of the anaerobiosis it becomes to all appearances normal. These observations have been made by at least two workers in the past, but there appears to be no published account in scientific literature.

The anaerobic existence of the cockroach provides a suitable set of conditions for the study of the energy-producing reactions which replace the oxidation process during oxygen lack.

In the present work an attempt has been made to measure the oxygen used by the cockroach under normal circumstances, and after a period of oxygen lack. In particular it was desired to show whether the cockroach incurs an oxygen debt as the result of anaerobiosis, similar to that produced by violent exercise in man.

Method.

Measurements of three types are required for the solution of the problems under consideration.

(1) It is necessary to know the oxygen intake of the cockroach under normal conditions and after anaerobiosis. This measurement must be made in air, the carbon dioxide being absorbed during the experiment.

(2) In order to obtain an approximate value for the respiratory quotient the difference between the volume of oxygen used and the carbon dioxide evolved must be known. This necessitates the measurement, in air, of the total change in the volume of the gases in which the cockroach is living.

(3) The measurement of the increase in volume of the gas in which the cockroach is living, due to the evolution of carbon dioxide during anaerobiosis.

The apparatus used resembled that designed for blood-gas analysis by Haldane [1920]. This gives readings of volume changes at constant pressure, and by the use of a compensating bulb avoids correction for small changes in temperature and pressure during the experiment.

The bulbs used to contain the cockroaches are shown in Figs. 1 and 2. The smaller type of bulb with only one chamber was used when the carbon dioxide was to be absorbed continuously throughout the experiment. It had the advantage that the air in the gas space could be rapidly replaced by nitrogen or *vice versa*. The larger type of bulb was used where the carbon dioxide was only absorbed during a portion of the experiment, the second bulb containing

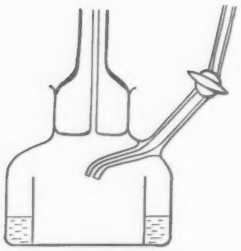

Fig. 1.

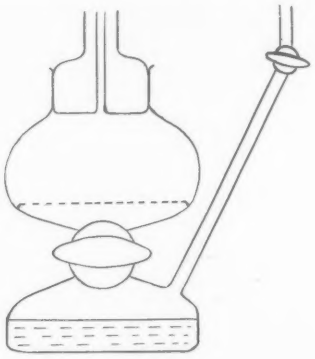

Fig. 2.

the potash being connected to the respiration bulb by means of a wide bore tap. In this apparatus a longer time had to be allowed for the nitrogen to replace completely the air in both chambers.

Except where it is otherwise stated all the experiments were carried out at 25°, the respiration bulb and its control being immersed in a thermostat which maintained that temperature within 0·05°.

In order to represent the results as uniformly as possible, they are calculated in mm.^{3} per g. weight of cockroach, per hour.

Variations in the insects used.

The insects used were *Periplaneta orientalis*, which can be caught at all seasons of the year in warm places such as a boiler house. The insects were stored in open beakers in a cupboard which was kept at 20°.

In the experiments described below a variety of specimens was used, particularly in the preliminary experiments designed to determine the normal metabolic rate. Body-weight and activity influenced the metabolic rate, and the diet the respiratory quotient.

Influence of body-weight on the metabolic rate.

A series of experiments was carried out on cockroaches, which were as nearly as possible uniform in all other respects save that of body-weight. The results are shown on the accompanying graph (Fig. 3) in which the weight in each individual experiment is plotted against the oxygen intake per g. per hour, measured in mm.³ It will be seen that the results lie upon a rough curve, showing a rapid fall in metabolic rate as the body-weight increases from 0·08 up to 0·2 g. From this point onwards increase in body-weight does not materially affect the oxygen uptake. It is necessary to mention at this point that the weight of a cockroach is very small, a specimen measuring about an inch in length rarely weighs more than 0·3 g.

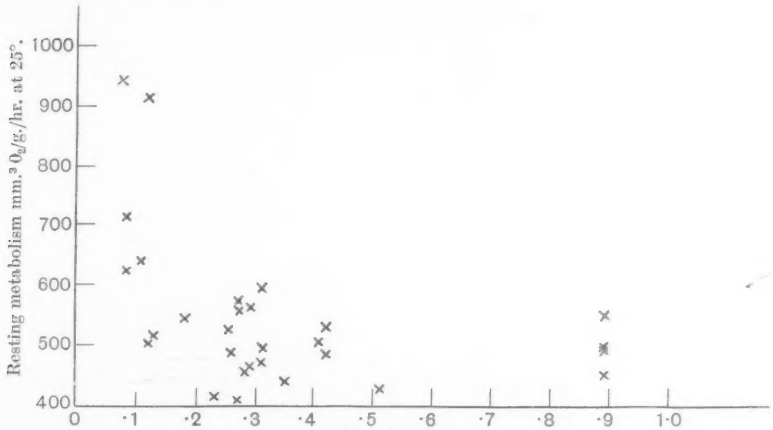

Fig. 3.

It will be noticed that as the weight increases the metabolic rate becomes more uniform; thus the percentage mean deviation for fifteen experiments with insects between 0·15 and 0·4 g. was 10·5, whilst that for nine insects between 0·4 and 1·0 g. was only 6·6. These deviations are, however, too great to assume a mean value for the anaerobic experiments, so that in each experiment of this type the normal oxygen uptake was determined for the individual cockroach used.

Influence of activity on metabolic rate.

Changes in activity during an experiment produced very anomalous results. It was found that if the movement was restricted to the antennae and the mandibles, no great change in the oxygen uptake took place, but if the cockroach moved about the vessel the uptake increased rapidly. Fortunately, only in rare cases did the insects show movement of this kind after they had once become settled in the bulb. Where marked movement was observed during the readings the experiment was abandoned.

Influence of diet on the respiratory quotient.

In order to determine the respiratory quotient the normal oxygen uptake was first measured. The tap connecting the respiration chamber with the potash bulb was then closed and the total change in volume measured. If it be assumed that this total change of volume is the sum of the volume changes of the oxygen and the carbon dioxide, it is possible, by subtracting the normal value for oxygen intake, to obtain a value for the carbon dioxide output, and hence for the respiratory quotient.

The investigations which have been made on these lines have not proved very satisfactory, owing to the difficulty which is experienced in ascertaining when the cockroaches have eaten. The mere presence of available foodstuff in the beakers with the insects cannot be taken as evidence. In some cases the cockroach was seen to begin to eat at once, whilst in others a cockroach which has been starving for over a week would not take any food at all. As a result of this, five determinations of the respiratory quotient of animals kept with food varied between 0·72 and 1·0. It is of interest, however, that five similar determinations on starving cockroaches all lie between 0·75 and 0·85, suggesting that the quotient is depressed by starvation in the same way that it is in the higher animals.

Metabolism in nitrogen.

For each experiment a cockroach was selected, which was as nearly normal as possible. It was placed in a bulb with air, potash being present to absorb the carbon dioxide, and the normal metabolic rate was determined. The air was then swept out by nitrogen as quickly as possible, and the cockroach kept under anaerobic conditions for a definite time, usually from half to one hour. At the end of this period the nitrogen was replaced by air, and the oxygen uptake following the anaerobiosis measured, until a normal oxygen uptake was again registered.

In the majority of the experiments the carbon dioxide was absorbed continuously throughout the experiment; under these conditions the gas volume during the anaerobic phase was constant within the limits of normal experimental fluctuations. If, however, the carbon dioxide is not absorbed whilst the nitrogen is present, an increase in volume is noted. This confirms the observations of Spallanzani [Foster, 1924] on snails, where carbon dioxide is evolved during oxygen lack. This carbon dioxide is probably due to the action of the lactic acid formed on the bicarbonates of the tissues.

The accompanying curves (Fig. 4) represent the oxygen uptake during two typical experiments. The continuous line gives the actual oxygen absorbed. The lower portion AB shows the normal uptake of the cockroach at the beginning of the experiment. At the point B nitrogen is introduced, and the uptake ceases until the point C is reached, when air is readmitted. The dotted line BB' shows the oxygen which it may be assumed would have been used if there had

been a continuous supply, whilst *CC'* indicates the oxygen which the cockroach would require, if it commenced to respire at the normal rate when the nitrogen was removed. It will be seen from the curves that when air is readmitted the cockroach takes up more oxygen than it normally requires, until an excess has been retained, which is equal to the volume the cockroach would have used during the time it was deprived of oxygen. The following examples illustrate this more definitely.

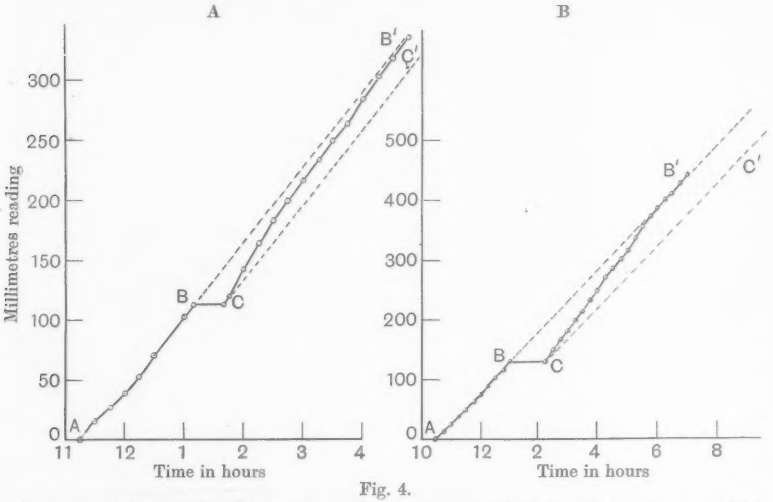

Fig. 4.

Experiment No. 44. Animal No. 19. Weight 0·31 g. Time of anaerobic existence = ½ hour. Time of recovery = 2½ hours (?). Temperature 25°.

Experiment No. 56. Animal No. 45. Weight 0·29 g. Time of anaerobic existence = 1½ hours. Time of recovery = 3¾ hours. Temperature 25°.

Exp.	Duration of anaerobiosis (hours)	Calculated oxygen debt (mm. reading)	Excess oxygen in ½ hr. (mm. reading)	1 hr.	1½ hrs.	2 hrs.	3 hrs.	4 hrs.
1	0·5	15	7	11	14	16	16	—
2	0·5	14	5	9	14	18	—	—
3	0·5	29	16	21	27	27	—	—
4	1	43	—	14	—	33	43	45
5	1	35	—	20	—	26	35	—
6	1·25	67	—	17	—	40	64	72

It is concluded from these results, that when the cockroach exists anaerobically it goes into debt for oxygen, in a manner similar to that which has been shown in man performing heavy physical work.

Discussion of the results.

The earlier experiments dealing with the relation between body-weight and metabolic rate, and between period of starvation and the respiratory quotient, are of interest primarily as controls, but they further serve to indicate the

general similarity between the metabolism of the cockroach and the higher animals. It is interesting to find in a cold-blooded animal like the cockroach the relation between body-weight and metabolic rate, but this may be accounted for by the fact that the majority of the smaller animals were very young, and that the phenomenon here observed is intrinsically one of age and not of body-weight. Some support is lent to this view by the fact that there is little variation in the metabolic rate after a definite weight (*i.e.* age) has been attained.

The power to go into debt for oxygen is of interest, and particularly, the close agreement between the volume of the debt and the volume of oxygen which would have been used during a time equal to the period of anaerobiosis. These observations suggest that the reaction producing energy under anaerobic conditions consists of the breakdown of one compound to another, which, though it has a lower energy content, requires an equal quantity of oxygen for its complete combustion. Such a reaction is the glycolytic formation of lactic acid, which supplies the energy in the anaerobic phase of muscular contraction. That a similar process, and not some entirely strange reaction, supplies the energy in the anaerobic existence of the cockroach is suggested as a working hypothesis.

This hypothesis is further supported by the similarity of the curves given above to those obtained by A. V. Hill and his co-workers on man. The only way in which these curves differ is in the time of recovery, which for the cockroach is much longer than for man.

The research is now being continued by an attempt to estimate the glycogen and lactic acid present in cockroaches before and after anaerobiosis, and by a further study of the respiratory quotients under these conditions. It is hoped by these means to establish definitely that in the case of the cockroach anaerobiosis is maintained by the same mechanism as that which supplies the energy for the anaerobic phase in the contraction of mammalian muscle.

Summary.

(1) The relation between body-weight and oxygen usage in the cockroach (*Periplaneta orientalis*) has been investigated: also the relation between starvation and the respiratory quotient.

(2) It has been shown that the cockroach whilst living in nitrogen goes into debt for oxygen, and that when the cockroach is returned to air an excess of oxygen over the normal uptake is consumed until the debt has been discharged.

The expenses of this research have been largely defrayed by a grant from the Royal Society.

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CXLVIII. A NOTE ON THE DISTRIBUTION OF REDUCING SUGAR AND THE MODE OF GLYCOLYSIS IN HUMAN BLOOD.

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(*Received October 11th, 1926.*)

Published work on the distribution of the blood sugar between the corpuscles and plasma shows very divergent results.

According to several authors, including Czaki [1922], Falta and Richter-Quittner [1922] and van Creveld and Brinkman [1921], the reducing sugar is confined to the plasma. Bönniger [1921], Braun [1922], Cristol and Danitch [1924] and Högler and Ueberrack [1924], on the other hand, are among those who have come to the conclusion that the corpuscles contain a considerable amount of reducing sugar.

In view of the present uncertain state of affairs regarding the nature and mode of existence of the reducing sugar in blood the method of estimation must be of considerable importance in work of this kind.

The most reliable methods are, according to the experience of the author, those based on the reduction of alkaline cupric salts, and of these that of MacLean is considered to be the most accurate as well as the most convenient.

This method was found to give results accurate to the third decimal place with standard solutions of glucose of concentrations of 0·050 % to 0·020 %. On comparison with the widely used picric acid method of Benedict, using blood, the method gave results almost invariably lower than the latter by two or three figures in the third decimal place. This difference was probably due to traces in the blood of creatinine, which is also estimated by Benedict's method.

Using MacLean's method, it was found that a sample of blood laked with 1 cc. of water and diluted with a correspondingly decreased volume of a more concentrated acid sodium sulphate solution gave results identical with those from a sample estimated in the usual way.

This comparison was considered necessary in view of the statement of Högler and Ueberrack [1924] that haemolysis of blood produced high results. Information regarding the method used by these authors is not to hand.

Van Creveld and Brinkman [1921] suggest that the distribution of the blood sugar is influenced by the early stages of coagulation and Cristol and Danitch [1924] consider the distribution to be affected by the anticoagulants usually

employed. The latter view is also held by Czaki [1922] who states that defibrination or the addition of anticoagulant renders the red cell membrane more permeable to glucose.

Owing to the fact that many of the original papers on the distribution of blood sugar referred to are not to hand, a critical review of the technique employed is not possible, but from the information available it is inferred that the following factors might account in some part at least for the conflicting results obtained.

(1) The use of different methods of sugar estimation.

(2) The laking or otherwise of the blood prior to the estimation.

(3) The influence of the anticoagulant when employed.

(4) The influence of incipient coagulation when no anticoagulant was used.

(5) The variation in the time elapsing between the withdrawal of the blood and the estimation of its sugar content, thus permitting the diffusion of a variable amount of sugar through the red cell membrane.

The distribution of the reducing sugar in human blood was consequently determined according to the following technique.

About 12 cc. of venous blood were collected through a paraffined needle into a cooled sterile paraffined test-tube. A sample of this was immediately pipetted off for estimation, and, after about 8 cc. of the blood had been poured into a graduated centrifuge tube in which one drop of 10 % potassium oxalate solution had been evaporated, this and the remainder of the blood were centrifuged and a sample of plasma pipetted from the untreated blood for estimation after 20 seconds.

The oxalated blood was centrifuged for about 3 hours, after which the relative volumes of corpuscles and plasma were noted. The earlier part of the procedure was carried out as rapidly as possible, but with due care, and in most cases the filtration of the sugar solution had commenced about 2 minutes after the withdrawal of the blood.

Capillary blood was obtained in quantity by washing the hands in warm water, drying, and, after swinging the arm round vigorously for some time and then ligaturing the base of the thumb, pricking the latter with a clean spear-pointed needle. The blood was collected and dealt with in the same way as the venous blood. In none of the experiments recorded did any trace of clotting occur. The percentage of glucose in the corpuscles was determined indirectly from the data obtained.

Results.

Untreated venous blood.

Exp.	1	2	3	4	5	6	7
% Reducing sugar in							
(1) Plasma	0·082	0·088	0·083	0·080	0·095	0·116	0·077
(2) Corpuscles	0·092	0·088	0·109	0·108	0·113	0·148	0·091
(3) Whole blood	0·087	0·088	0·095	0·093	0·104	0·132	0·084
% of corpuscles	51	52	48	45	51	50	49

Untreated capillary blood.

Exp.	1	2	3	4	5	6	7	8	9
% Reducing sugar in									
(1) Plasma	0·073	0·086	0·103	0·100	0·087	0·099	0·124	0·088	0·099
(2) Corpuscles	0·089	0·063	0·075	0·079	0·103	0·087	0·102	0·064	0·087
(3) Whole blood	0·081	0·075	0·090	0·090	0·095	0·093	0·113	0·077	0·093
% of corpuscles	51	52	46	48	51	50	49	46	50

Although the blood was drawn at varying intervals after the previous meal, and from a number of individuals, in no case were the corpuscles found to be sugar-free. In fact all but one of the samples of venous blood showed more reducing sugar in the corpuscles than in the plasma.

In the capillary blood, on the other hand, the distribution was more variable, seven of the nine samples estimated showing more sugar in the plasma than in the corpuscles.

A comparison of the sugar distribution in the fresh blood with that in the oxalated sample confirmed the opinions previously referred to regarding the influence of anticoagulant in the permeability of the red cell to sugar. Owing, however, to the onset of clotting, comparisons could not be made at such an interval after withdrawal as would allow an appreciable amount of diffusion.

Exp.	1		2		3		4	
% Reducing sugar in	Untreated blood	Oxalated blood	Untreated blood	Oxalated blood	Untreated blood	Oxalated blood	Untreated blood	Oxalated blood
(1) Corpuscles	0·109	0·100	0·106	0·106	0·084	0·072	—	—
(2) Plasma	0·083	0·086	0·080	0·080	0·106	0·100	—	—
(3) Whole blood	0·096	0·093	0·092	0·092	0·095	0·086	0·101	0·095
Time elapsing after withdrawal ...	3 mins.		2 mins.		12 mins.		5 mins.	

Estimations made with oxalated blood a considerable time after removal did not, as would be expected, always show an equal distribution of sugar between the corpuscles and plasma, the percentage of sugar in the corpuscles now being relatively high as compared with that in the plasma.

Exp.	1	2	3	4	5	6	7	8
Time after withdrawal	mins. 12	mins. 30	hrs. 16	hrs. 24	hrs. 24	hrs. 30	hrs. 48	hrs. 72
% Reducing sugar in								
(1) Whole blood	0·081	0·079	0·077	0·046	0·045	0·071	0·078	0·041
(2) Corpuscles	0·074	0·090	0·108	0·057	0·045	0·066	0·094	0·046
(3) Plasma	0·089	0·100	0·039	0·035	0·045	0·079	0·062	0·036

This might have been due, as suggested by MacLeod [1921], to a difference in the solubilities of sugar in the plasma and corpuscular materials, but it suggested that glycolysis might be more rapid in the plasma than in the corpuscles, a view that would be in accordance with the more rapid glycolysis observed in oxalated blood. To test this hypothesis samples of oxalated blood and corpuscle-free plasma separated from the latter were kept in sealed sterile tubes for varying periods, at the end of which the sugar in the separated plasma,

that in the plasma of the whole blood separated just before the estimation, and that in the whole blood itself were determined.

Exp.	1		2		3	
Time after withdrawal	2 mins.	16 hrs.	2 mins.	24 hrs.	2 mins.	48 hrs.
% Reducing sugar in						
(1) Whole blood	0·092	0·077	0·084	0·046	0·103	0·078
(2) Corpuscles	0·107	0·107	0·085	0·057	0·106	0·094
(3) Plasma	0·081	0·039	0·083	0·035	0·100	0·062
(4) Separated plasma	—	0·077	—	0·072	—	0·100

Exp.	4		5		6		7		
Time after withdrawal	mins. 10	hrs. 72	mins. 2	hrs. 24	hr. 1	hrs. 24	hrs. $1\frac{1}{2}$	hrs. $5\frac{1}{2}$	hrs. $28\frac{1}{2}$
% Reducing sugar in									
(1) Whole blood	0·086	0·041	0·084	0·046	0·081	0·045	0·083	0·067	0·049
(2) Corpuscles	0·100	0·046	0·091	—	0·085	0·045	0·084	—	—
(3) Plasma	0·100	0·036	0·077	—	0·077	0·045	0·082	—	—
(4) Separated plasma	—	0·084	—	0·063	—	0·070	—	0·082	0·081

Exp.	1	2	3	4	5	6	7
Decrease of glucose in							
(1) Whole blood	0·015	0·038	0·025	0·045	0·038	0·036	0·034
(2) Corpuscles	0	0·028	0·012	0·054	—	0·040	—
(3) Plasma	0·042	0·048	0·038	0·064	—	0·032	—
(4) Separated plasma	0·004	0·011	0·000	0·016	0·014	0·007	0·001

In the separated plasma the glycolysis, when it occurred, was in all cases very slight as compared with that in the whole blood, the decrease in the sugar of the latter occurring chiefly in the plasma it contained. This suggests that the glycolysis is due almost entirely to the activity of leucocytes and in a varying degree to other factors. The rate of disappearance of sugar (5–6 mg. of sugar per g. of leucocytes per hour) does not seem too high to be accounted for mainly by the metabolic activity of leucocytes.

Summary.

(1) In human blood the red corpuscles contain a considerable amount of reducing sugar. In capillary blood there is generally more sugar in the plasma than in the corpuscles, whereas in venous blood the reverse is generally the case, owing to the plasma sugar being more accessible to the tissues.

(2) From the results obtained it is inferred that the glycolysis in human blood is due almost entirely to the activity (chiefly metabolic) of the leucocytes.

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CXLIX. ON THE DENATURATION OF EGG-ALBUMIN BY WEAK ACIDS IN THE PRESENCE OF SALTS.

By HAROLD MASTIN and SAMUEL BARNETT SCHRYVER.

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(*Received October 11th, 1926.*)

This work arose out of an attempt to prepare a salt-free solution of the proteins of egg-white by means of electrolysis. It was soon found that egg-albumin itself was altered under the conditions of the experiment, and a substance was obtained which did not correspond in properties with any of the known denaturation products of albumin. It was thought, originally, that the change was due to the electric current itself, but subsequent investigation showed that the change was due to the joint action of the salts present, and the acid formed as a result of the electrolysis. The material may therefore be regarded as a stage in the denaturation of albumin by acids, and is of great interest, in that it is possible to obtain evidence as to its structural relationships to fresh albumin on the one hand, and to more fully denatured albumin on the other hand.

In the earliest experiments the egg-white was diluted with two volumes of water, beaten up and centrifuged to remove particles of membrane, etc. It was then introduced into an ordinary lamp glass, the bottom end of which was closed by muslin coated with collodion to form a semi-permeable membrane. A short length of wide glass tube, closed at the lower end in a similar manner, dipped into the liquid from above. This tube and a beaker surrounding the lower end of the lamp glass formed the electrode compartments of a tripartite cell, the lamp glass containing the albumin being the centre section. Under normal conditions the electrode compartments contained distilled water which could readily be removed in order to examine the substances passing through the membranes. A platinum anode was used at the top and a platinum or mercury cathode in the beaker: the membranes were about 15 cm. apart and 50 to 200 volts were used according to the conductivities of the solutions. It was expected that as soon as the electrolytes were removed, the globulin would be precipitated, leaving the albumin, "conalbumin" and ovomucoid for further treatment, but it was soon found that the precipitate was not entirely composed of globulin. The next step was to remove the globulin by half saturating the egg-white with ammonium sulphate, dialyse out most of the electrolyte and then submit the residue to electro-dialysis. When this was

They are insoluble in water, soluble in dilute alkalis, including ammonia, and are reprecipitated from alkaline solution by acids, but if the temperature rises at all during the neutralisation they become insoluble in alkalis. In some cases, particularly when electrolysis has been prolonged, there is a small part which is not soluble in ammonia. The freshly prepared material dissolves readily, giving a perfectly clear solution. It is not soluble in dilute acids in the cold, but is soluble with alteration in $N/25$ HCl at 37°. After solution at 37° the substance has the properties of an acid albumin, only being reprecipitated round the isoelectric point. All samples of the insoluble product showed an intense nitroprusside reaction. Within the limit of experimental error, the Hausmann numbers of the insoluble products were identical with those of crystalline egg-albumin and "conalbumin."

Action of ferments on acid protein.

The effect of trypsin and pepsin on the acidic precipitated protein was examined. Liquor pancreaticus (Benger) was used for the tryptic titration. The curves (see Fig. 1) for the rate of digestion were identical for the altered product and coagulated albumin, while unheated albumin showed a slower rate initially but the final result was the same. It was difficult to examine the effects of pepsin as the substance dissolved in the hydrochloric acid used for the digestion. Some comparison could be made by using extremely dilute acid and comparing the turbidities in the usual way.

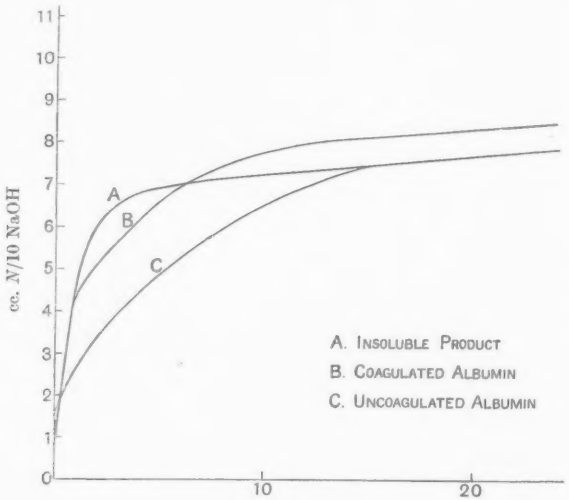

Fig. 1.

The results are given in tabular form, the amount of pepsin being in arbitrary units.

Time in hours HCl	Pepsin	Coagulated 1·9	2·5	3	21	Altered albumin 0·2	0·4	0·7	1·0	1·9	2·5	3	21
	10	+ + +	C	—	—	+ + +	+ + +	C	—	—	—	—	—
N/100	5	+ +	C	—	—	+ + +	+ + +	+ + +	C	—	—	—	—
	2	+	+ +	—	C	+ + +	+ + +	+ + +	C	—	—	—	—
	0		No action			—	+	+	+	+ +	+ +	+ +	+ +
	10	—	—	—	+ +	—	—	+	+	+	+ +	+ + +	C
N/250	5	—	—	—	+ +	—	—	+	+	+	+ +	+ + +	C
	2	—	—	—	+	—	—	+	+	+	+ +	+ + +	C
	0		No action			—	—	—	—	—	+	+	+
	10	—	—	—	+	—	—	—	+	+	+	+ +	C
N/500	5	—	—	—	+	—	—	—	+	+	+	+ +	C
	2	—	—	—	+	—	—	—	—	+	+	+ +	+ + +
	0		No action			—	—	—	—	—	—	—	+

C = complete

These results indicate that the acidic protein is more readily digested than coagulated protein.

Analysis and preparation of products.

Crystalline egg-albumin was prepared in the usual manner from 820 cc. of egg-white. It was then recrystallised twice and its solution dialysed for 11 days in running distilled water. It then contained 0·05 % of its nitrogen in the form of ammonia. It filtered clear (preparation I (*a*)). The coagulated albumin was prepared by heating a dilute solution of albumin for 20 minutes (preparation I (*b*)). 200 cc. of a solution of crystallised egg-albumin containing 4·4 mg. of protein N and 3·6 mg. of ammonium sulphate N per cc. were electrolysed for 2 days using 100 milliamps. during the day and 50 at night. The insoluble precipitate which formed was centrifuged off, washed thoroughly on the centrifuge and suspended in 100 cc. of water (preparation III B (*a*)). One part of the suspension was directly analysed and another part was heated for half an hour on the water-bath and then analysed (preparation III A (*b*)).

After centrifuging off the precipitate, saturated ammonium sulphate solution was added to the slightly turbid liquid, when a bulky gelatinous precipitate was formed, which occupied half the volume even after centrifuging. It did not dissolve quite completely in water and its solution was dialysed till free from ammonium sulphate and then filtered. In the table below it is referred to as "first fraction of albumin not precipitated during electrolysis with ammonium sulphate" (preparation II (*a*)). The greater part of the protein could be precipitated from this solution by 45 % saturation with ammonium sulphate. From the filtrate of this precipitate a second fraction could be obtained on addition of more ammonium sulphate. It did not form a coherent mass on centrifuging and was readily soluble in water. The aqueous solution was dialysed (preparation II (*b*)).

Experiments were also carried out to determine the effect of salts other than ammonium sulphate on the production of the acidic protein. For this purpose two exactly similar solutions of crystallised egg-albumin were electro-

lysed in series overnight to remove last traces of ammonium sulphate, and then one was made $M/10$ with potassium chloride and the other $M/10$ with ammonium sulphate. The electrolysis was continued with a current of 50–100 milliamps. The two liquids behaved differently when submitted to electrolysis. After 20 hours the solution containing ammonium sulphate gave a precipitate, which was centrifuged off. 5 cc. of the clear liquid required 3 cc. sodium hydroxide to change methyl red. The solution containing the potassium chloride, however, showed only a faint turbidity. 5 cc. required 2·5 cc. $N/10$ alkali for neutralisation to methyl red, and at the neutral point a heavy precipitate came down, which dissolved in excess of alkali. On continuing the electrolysis, a second precipitate was formed in the ammonium sulphate solution. No precipitate was formed in the experiment with potassium chloride, but on addition of half the volume of $4N$ potassium chloride, practically the whole of the protein was precipitated. The precipitate behaved exactly like the insoluble precipitate previously obtained, being soluble in dilute alkalis, insoluble in dilute acids and showing a strong nitroprusside reaction (preparation III A (*c*)). Another sample of salt-free albumin was electrolysed in the presence of $M/10$ sodium chloride. A flocculent precipitate formed overnight. The analyses of this sample are given with reserve as free chloride was found in the solution (preparation III B (*b*)).

Another sample of albumin solution free from electrolytes was electrolysed for 20 hours. No precipitate had formed and the clear solution was submitted immediately to analysis (preparation II (*c*)).

Preparations as numbered in text	cc. $N/10$ KOH	COOH groups	% amino-N	NH_2 groups	Nitro-prusside reaction
I. (*a*) Crystalline albumin	2·39	25	4·14 4·44	16 17	–
(*b*) Coagulated albumin	2·25	24	5·39	20	–
(*c*) Conalbumin	2·57	27	5·52	21	–
II. (*a*) First fraction of albumin not pptd. during electrolysis with $(NH_4)_2SO_4$	3·55	38	4·84 5·57	18 21	–
(*b*) Second fraction ditto	3·51	37	—	—	–
(*c*) Albumin electrolysed without electrolytes	3·55	38	—	—	+
III. A (*a*) Pptd. with $M/5$ $(NH_4)_2SO_4$ $N/5$ H_2SO_4 in the cold	4·16	44	3·42	13	+
(*b*) Electrolysed with $(NH_4)_2SO_4$ and heated ½ hour	4·13	44	—	—	+
(*c*) Electrolysed with KCl	4·48	48	3·61	14	+
B (*a*) Electrolysed with $(NH_4)_2SO_4$ ...	5·42	58	4·20* 4·66	16 18	+
(*b*) Electrolysed with NaCl	5·52	59	—	—	+
(*c*) Conalbumin electrolysed with $(NH_4)_2SO_4$	5·81	62	—	—	+

* Is a different sample prepared at a different time.

Analysis. The solutions or suspensions were examined for free amino-groups by the ordinary Van Slyke method. Two portions of 10 cc. were each shaken for ¾ hour before the total nitrogen evolved was measured. The free amino-nitrogen is expressed as a percentage of the total and also as free

amino-groups per molecular aggregate assuming Sørensen's value of 34,000 and 380 nitrogen atoms. The free carboxyl groups (or other acidic groups) were estimated directly by the formaldehyde titration method or by direct titration in alcoholic suspension. The titration in alcoholic suspension using $N/10$ alcoholic potash gave slightly lower readings, but the figures were relatively the same. In the formaldehyde titration 45 cc. of solution or suspension containing 50 mg. of protein nitrogen were mixed with 40 cc. of formalin diluted with an equal volume of water. The results are given as cc. of $N/10$ alkali for 50 mg. of nitrogen and also calculated as free carboxyl groups per molecular aggregate. The nitroprusside reaction was carried out as described by Harris [1923]. It was not found possible to determine the free —SH groups quantitatively as the ordinary oxidation methods are not applicable owing to the insoluble character of the substances and to the fact that the large molecular aggregates prevent quantitative conversion to the disulphide owing to steric factors.

Discussion.

The number of free carboxyl groups divides the substances examined into three groups:

I. 24–27 free carboxyl groups and negative nitroprusside reaction; includes crystalline albumin, coagulated albumin and conalbumin.

II. 37 or 38 free carboxyl groups and negative nitroprusside reaction; includes the three materials which have been electrolysed but not converted into an insoluble form: this group is not due to admixture with members of group III, but must be regarded as a definite intermediate stage as there is no nitroprusside reaction.

III. Characterised by the nitroprusside reaction and by the fact that all members are insoluble in water and dilute acids. It can probably be subdivided into two sub-groups, the one containing 44 to 48 carboxyl groups and the other 58 to 62. The latter is probably only a later stage of the former. The three fully electrolysed products form the second class (sub-group III (a)) and the first is composed of the material obtained by acid and salt without electrolysis on long standing, a fully electrolysed material heated on the water-bath and also the material electrolysed with potassium chloride which did not precipitate or become insoluble until after salting out with more potassium chloride. There are thus nearly two and a half times as many free carboxyl or other acid groups in the denatured albumin as in the original crystalline albumin.

The values for the free amino-groups exhibit no regularities and with two exceptions lie between 16 and 21. The experimental error in these experiments is great owing to the difficulty of transferring the suspensions completely to the apparatus, and the error is greatest with the denser precipitates in group III, since a relatively larger proportion cannot be washed into the apparatus. Both the low results, 13 and 14, were obtained with dilute suspensions, when

the error was even more serious. Whether there is any closing up of NH_2 groups or not, it is quite clear that the increase in free carboxyl or other acidic groups is not due to the breakdown of peptide linkings which would be accompanied by an *increase* in amino-groups. The latter stage of the change is certainly accompanied by the unmasking of free thiol groups and the process may be formulated

that is, as the breaking down of a thiol-depside ring. It is not possible to say whether the free thiol group would behave as an acidic group and be titrated in the formaldehyde titration as it would depend on the other parts of the molecule, but it is probable that it would act in this way. Thioglycollic acid is a normal dibasic acid and forms normal salts $NaS.CH_2.COONa$. The members of group III show as many as 22 more free acid groups than group II and this would require 11 thiol groups to be liberated, *i.e.* equivalent to 1 % of the total albumin as thiol sulphur. The cystine in albumin is insufficient to account for this quantity and it would have to be derived from the unidentified sulphur in the albumin. Harris considered this explanation of the production of the free thiol groups in denatured protein but inclined to another formulation; in the present case only the one explanation is valid. It is not a complete explanation as it does not take into account the formation of group II from group I and the opening up of ten acid groups. It is an interesting speculation to imagine that this is due to the opening of similar depside rings and liberation of hydroxyl groups instead of thiol groups. The rapid increase in the number of hydroxyamino-acids which have been isolated is of interest in this connection and further it is known that tyrosine is one of the first, if not the first, amino-acid to be liberated from albumin under the influence of enzymes. The natural extension of this work to acid denatured proteins is being carried out in these laboratories.

It has been found that similar acidic substances, yielding the nitroprusside reaction, are formed when egg-albumin is denatured at the interfaces of aqueous egg-albumin solutions and fluids such as chloroform which are immiscible with water, the action of which is very specific. The work on these phenomena will form the subject of a later communication.

Summary.

(1) A well-defined acidic denatured product of albumin has been obtained in various ways. It is soluble in dilute alkalis and insoluble in dilute acids.

(2) The formation of this substance involves an increase in the number of free acid groups without corresponding increase in the amino-groups. At the same time free —SH groups appear.

(3) Evidence is produced to show the presence of a new type of linkage in proteins. A *thiodepside* or C—CO.S—C— linkage breaks down to give the corresponding carboxyl and thiol groups.

The above work was carried out under the auspices of the Adhesives Committee of the Department of Scientific and Industrial Research.

REFERENCE.

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CL. THE CONSTITUTION OF SPONGES.

I. THE COMMON BATH SPONGE, *HIPPOSPONGIA EQUINA*.

By VERNON JAMES CLANCEY.

(*Received August 3rd, 1926.*)

Introduction.

It is the belief of the author that the constitution of spongin varies with the source of its origin, and that the substances which form the main framework of the Euceratosa and which surround the spicules in the Pseudoceratosa are not identical, as appears to have been assumed by many physiologists. It is with the object of proving or disproving this theory that the present series of researches has been undertaken.

Many workers have published results for the constitution of spongin, usually obtained from either *Hippospongia equina*, the common bath sponge, or *Euspongia officinalis*, the "Turkey cup sponge." These results show remarkable differences due to defective methods of analysis and working with commercial sponges that have been variously macerated and bleached.

In this paper the author presents the results that he has obtained by applying the modern methods of protein analysis to the spongin from *H. equina*.

Theoretical and Methods.

The spongin for the following estimations was prepared by washing successively with water, dilute acid and alkali and then drying in a vacuum over P_2O_5. This gave a substance containing 1·25 % ash and 0·8 % water.

The cause of the differences in the results of previous workers appears to be the presence of the iodine complex. Wheeler and Mendel [1909] isolated this complex from a barium hydroxide hydrolysis and showed that it was identical with the iodogorgonic acid first obtained from *Gorgonia cavolinii* by Drechsel [1896], the structure of which was shown to be that of 3 : 5-di-iodotyrosine by Wheeler and Jamieson [1905], and Wheeler and Johns [1910]. They also showed that it does not give all the reactions usually associated with tyrosine; for example, it gives the xanthoproteic reaction owing to the presence of the benzene ring, but the iodine in the ring apparently prevents it from reacting with Millon's reagent.

In order to obtain complete hydrolysis and comparative values for the amino-acids, the following method was adopted.

A weighed portion of the spongin, about 3 g., was introduced into boiling 20 % hydrochloric acid and boiled under a reflux for about 30 hours until no further increase of the amino-acids in the solution was shown. It is necessary to raise the acid to boiling before adding the protein in order to obtain concordant results [cf. Knaggs, 1923]. The resulting solution, after filtration from the "humin," was made up to 250 cc. A portion of 100 cc. was analysed by the methods of Van Slyke and Hausmann. The results show the discrepancies that arise by neglecting the iodine complex.

Another portion of the hydrolysed mixture was evaporated down, *in vacuo* at 40°, to about 1 cc. Water was added to bring it back to its original volume and the mixture again evaporated. This was repeated a third time to ensure that nearly all the hydrochloric acid had been removed. The resulting liquid was diluted to about 100 cc., and brought to boiling under a reflux condenser. A few drops of nitric acid were added, and then a solution of silver nitrate until no further precipitation took place. The liquid was filtered through a Gooch crucible and the precipitate well washed. The iodine in the precipitate was estimated by replacing it with chlorine and noting the loss of weight. A Kjeldahl determination was made to ascertain whether any of the nitrogen had been carried down with it. A control experiment showed that the addition of the nitric acid had no effect on the distribution of the nitrogen.

The filtrate from the silver precipitation was evaporated down and the nitrogen groups estimated in the ordinary way.

The total nitrogen was determined by Kjeldahl's method in an aliquot portion of the hydrolysed mixture.

The iodine was estimated by a combination of the methods of Kendall [1920], and Winkler [1915, 1916].

The individual amino-acids were estimated by the methods given in Plimmer's *Constitution of the Proteins*, using the "ester" separation.

Results.

Table I gives the values for the "Hausmann numbers" obtained for spongin by the above two methods, and comparative values for iodogorgonic acid prepared from tyrosine by the method of Wheeler and Jamieson [1905].

Table I.

Substance	Humin-N %	Amide-N %	Diamino-N %	Monoamino-N %	Total N %
Spongin—					
Without removal of iodine	0·03	1·12	2·39	12·58	16·12
With removal of iodine	0·03	0·98	2·34	12·77	16·12
Iodogorgonic acid	0·00	2·99	0·00	0·24	3·23

The above values are calculated as percentages of the total protein.

Table II gives the values for the nitrogen in eight groups, calculated as percentages of the total nitrogen.

Table II.

Group	Without removal of iodine %	With removal of iodine %
Humin-N	0·18	0·18
Amide-N	6·95	4·91
Cystine-N	0·04	0·02
Arginine-N	11·16	10·90
Histidine-N	0·00	0·00
Lysine-N	4·37	4·39
Monoamino-N	77·43	80·01
Non-amino-N	2·86	2·78
	102·99	103·19

The following are the values for the individual amino-acids obtained by the ordinary methods, the tyrosine being estimated in a sample after removal of the iodine. They are given as percentages of the protein.

Glycine	14·0 %	Aspartic acid	4·5 %
Alanine	0·2 %	Glutamic acid	18·4 %
Leucine	7·9 %	Tryptophan	0·0 %
Tyrosine	2·8 %	Arginine	5·9 %
Cystine	trace	Lysine	3·6 %
Proline	5·7 %	Histidine	0·0 %
Hydroxyproline	0·0 %	Ammonia	0·8 %

The iodine content of the spongin was 1·46 %, whilst the silver precipitation carried down 1·41 %, the latter value being low, probably owing to the difficulty of getting an accurate estimation.

Conclusions.

Spongin on acid hydrolysis yields, besides the other amino-acids, iodogorgonic acid. This is liable to be overlooked owing to the fact that its solubility is 0·2 g. in 100 g. of water and that it is not insoluble as stated by Henze [1903]. It does not give the Millon reaction as it contains an iodine atom in both the 3 and the 5 positions according to Hofmeister [1898], and Blum and Vaubel [1898].

The iodogorgonic acid yielded on hydrolysis is converted into tyrosine and silver iodide by the treatment with acid silver nitrate. The yield of iodine is equivalent to 2·1 % tyrosine, whilst 2·8 % was found. This yield is equivalent to 4·7 % iodogorgonic acid in the protein.

By first removing the iodine it is possible to estimate the tyrosine in the ordinary way and to obtain true values for the nitrogen distribution. The tyrosine appears to be totally present as iodogorgonic acid, and varies with the percentage of the iodine.

On hydrolysis with dilute alkali iodogorgonic acid yields practically all its nitrogen as ammonia, *i.e.* it behaves as an amide. Consequently the iodine must first be removed in order to obtain correct values for the amino-acids.

Spongin yields principally glutamic acid and glycine on hydrolysis, with smaller quantities of leucine, aspartic acid, arginine, proline, lysine and tyro-

sine. It does not appear to be very closely related either to the chitinoids or to fibroin, as has been suggested by some authors, but is rather more closely related to the collagens, especially as there is some evidence that on prolonged heating with water a portion goes into solution which is reprecipitated by alcohol.

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CLI. A MICRO-METHOD FOR THE DETERMINATION OF THE HAUSMANN NUMBERS OF PROTEINS.

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The Hausmann numbers, or the determination of the partition of nitrogen, should be a rapid and convenient method for establishing definite criteria for a particular protein. This analysis, however, originally due to Hausmann [1899] and modified by Osborne and Harris [1903], has been subjected to criticism on several scores. In particular, several workers have found the estimation of the diamino-nitrogen, or basic fraction, to give unreliable results, which cannot always be duplicated. This fraction has been shown [Osborne, Leavenworth and Brautlecht, 1908] to correspond approximately with the amounts of the diamino-acids present in the protein, yet the results obtained by different workers vary so widely that it is evident that the composition of the precipitate is purely empirical and depends to some extent on the conditions.

Apart from the criticisms which were put forward in the early stages, and which have been answered by Osborne, Gumbel, and others, there are five main sources of error in the method as usually practised. Certain of these errors being exaggerated when the analysis is performed on the micro-scale, it became necessary to eliminate them as far as possible.

1. Jodidi and Moulton [1919] showed that the precipitate of humin and magnesia, filtered off after determination of the amide or ammonia nitrogen, contained an amount of nitrogen roughly proportional to the amount of magnesia used. Thus, in experiments on caseinogen,

Weight of MgO added	4 g.	Nitrogen in the precipitate	4·78 %
„ „ „	2 g.	„ „ „ „	2·60 %
„ „ „	1 g.	„ „ „ „	1·72 %

This absorption of nitrogen by the magnesia causes a corresponding decrease in the basic and monoamino-nitrogen found in the filtrate. On the other hand, the insoluble humin precipitate, if filtered off separately, before addition of MgO, contains only 0·1 % of the total nitrogen. It is clearly incorrect to regard this MgO precipitate as representing humin nitrogen, since it consists mainly of soluble nitrogen adsorbed on to the precipitate. Moreover, it has been found that thorough washing with hot water does not reduce the nitrogen in the

precipitate to an appreciable extent, so that the adsorption on to the magnesia is of a fairly close nature. The conclusion reached by Jodidi and Moulton is that if 1 g. of magnesia be used throughout, satisfactory results can be obtained, and they ascribe Osborne and Harris' concordant results to this fact.

2. In common with other workers, Jodidi and Moulton have overlooked the important question of the *amount of magnesium remaining in solution*. This is, of course, independent of the amount added, but depends upon:

(*a*) the amount of HCl remaining in the liquid after the evaporation, *i.e.* the completeness of the removal of the acid,

(*b*) the amount of amino-acid hydrochloride to be neutralised,

(*c*) the volume of water, in which magnesia has a very slight solubility (about 1 : 55,000).

It will, however, be shown that the presence of a variable amount of magnesium considerably influences the values obtained for the diamino-nitrogen. Other salts, or an excess of sulphuric acid, have a similar effect in increasing the amount of precipitate.

In a series of experiments gelatin was hydrolysed in 15 % H_2SO_4, diluted to three times the volume, and precipitated with phosphotungstic acid in presence of various salts.

	% nitrogen in the precipitate.		
	(*a*)	(*b*)	Mean
Salts absent	13·2	—	13·2
1 g. $MgSO_4.7H_2O$ added	15·0	15·2	15·1
1 g. NaCl added	18·8	18·6	18·7
1 cc. (extra) H_2SO_4 added	17·0	17·3	17·15

In the same way considerable variations in diamino-nitrogen were found to correspond with the quantities of alkali needed to make the solution alkaline for the amide determinations:

	Diamino-N of gelatin %	Diamino-N of edestin %
1·5 cc. 10 % NaOH	14·6	27·8
2·0 cc. 10 % NaOH	15·7	29·4

Thus the amount of nitrogen in the phosphotungstic precipitate varies with the amount of salt present. The errors involved are considerable, and may go far towards explaining the difficulty found in obtaining reliable values for the diamino-nitrogen.

The process, occasionally adopted, of redissolving the MgO in H_2SO_4 before filtering off the humin is therefore open to objection on the score that it involves the addition to the filtrate of a variable amount of salt and acid. In the method described below, a constant amount of NaOH is added to the solution, so that the quantities of salt and acid introduced are the same throughout, while the error due to the magnesia precipitate is avoided. The values for the amide nitrogen obtained by the use of NaOH do not differ considerably from those found with MgO, though on the whole slightly higher (see results for gliadin), while the humin nitrogen (the true insoluble matter) is so low as to be negligible

in some cases, as in gelatin. The minute amount of flocculent precipitate can, in the case of gelatin, be allowed to remain since it increases the diamino-N by less than 0·1 %.

3. The need for absolute standardisation of the conditions was shown by Knaggs [1923], according to whom the protein must be hydrolysed immediately on adding the acid—preferably thrown into the boiling acid. Further, the liquid must not be allowed to stand with the 5 % H_2SO_4 before precipitating with the phosphotungstic acid, or if it has stood, it should be heated to boiling or autoclaved and precipitated immediately on cooling.

The author has found the most reliable results to be obtained in the micro-method by heating the 5 % acid solution in the autoclave, cooling quickly and precipitating.

4. The concentration of the solution is an important factor. In the first place, if the precipitate is small, the effect of solubility is large, while if the precipitate is large and bulky, it is difficult to wash completely on the filter, and hence it may contain non-basic nitrogen from the filtrate. Experiments in redissolving and reprecipitating the phosphotungstates to avoid this were unsuccessful on account of the large amount of alkali needed to dissolve the precipitate and the consequent alteration of the equilibrium in the second precipitation. The whole mechanism of the precipitation appears to be a mass action phenomenon and is continuously variable, except within a limited range of concentration, which depends on the particular protein taken. Below this concentration precipitation is incomplete, even after allowing for the solubility of the phosphotungstates.

It is therefore essential to use the same amount of protein for the hydrolysis in each case, as nearly as possible.

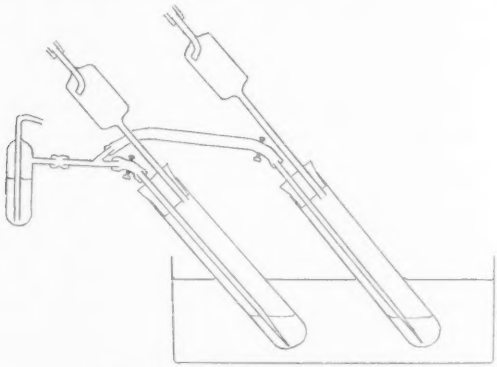
5. In addition, the temperature of the solution affects the precipitation, and while cooling to 20° is specified by Hausmann and by Osborne and Harris [1903], it has been found that 0° is a more convenient precipitating temperature for the purpose of this method.

Experimental.

The method is designed to deal with 3–6 mg. of total nitrogen in each determination, and hence for the hydrolysis some 15 mg. nitrogen should be used to allow two determinations. This corresponds to 90–100 mg. of dry protein, *i.e.* one-tenth he amount used in the ordinary method.

Hydrolysis. With dry material the protein powder is dropped into 30 cc. boiling 18–20 % HCl. Most of the experiments were carried out with 0·7 % gelatin solution (1 mg. N per cc.), in which case 15 cc. solution are poured into 15 cc. boiling 35 % HCl. The mixture is heated in a 100 cc. Kjeldahl flask under a reflux condenser for 18 hours. To avoid contamination by products dissolved from the rubber stoppers by the HCl, a hang-in glass condenser of simple design was used, large enough to fit closely into the neck of the flask, and through this water was circulated.

Evaporation of the acid. Two portions of 10 cc. each are pipetted out on cooling into hard glass boiling tubes (200 × 25 mm.), which are fitted with fine capillaries reaching almost to the bottom, provided with screw clips above to regulate the air passing through them (see diagram). Short exit-tubes are connected to small still-heads of oblong type (35 × 25 mm.), and thence to the pump. The two tubes are placed slantwise in a warm bath at 50°–60°, and the acid evaporated off under reduced pressure. The small amount of air which passes through the capillaries is brought through a bubbler of H_2SO_4 to remove ammonia. The liquid is brought as near dryness as possible—with a good pump in about 3 hours. Tubes and stoppers are then rinsed down with about 10 cc. of water, and a drop of phenolphthalein is added.



Apparatus for evaporation of the acid.

Amide nitrogen. 2 cc. of 10 % NaOH are then run in, followed by H_2SO_4 until just acid. 2 % NaOH is then added drop by drop until the indicator is just pink. The amide or ammonia nitrogen is then determined in an apparatus similar to that of Folin [1902] for determining ammonia in urine. In the first of three boiling tubes is placed H_2SO_4, in the second the alkaline liquid, while the third, which is fitted with a bubbler, contains 5 or 10 cc. $N/70$ H_2SO_4 to absorb the ammonia. The second tube is fitted with a small still-head to prevent loss, and is immersed in water at 40°–50°, and air is aspirated briskly through for 90 minutes. The receiving tube and bubbler are rinsed down, washed out into a small flask, and the solution is titrated at once with $N/70$ NaOH, methyl red being used as indicator.

Humin nitrogen. The solution is made acid with one drop of H_2SO_4, and the insoluble dark precipitate centrifuged off on a small centrifuge. 15 cc. urine tubes with pointed ends were found to be most satisfactory, using a water-driven centrifuge. In some cases the humin may be neglected if the liquid is clear and pale; otherwise the precipitate is washed three times with 1–2 cc. of water, centrifuging for 5–10 minutes between each washing. It is transferred with a few drops of water to a hard glass test-tube, and incinerated

with H_2SO_4 and potassium and copper sulphates, and the nitrogen estimated by Parnas and Wagner's [1921] modification of Pregl's method, which is used for all subsequent nitrogen estimations.

Diamino-nitrogen. 0·3 cc. concentrated H_2SO_4 is now added to the filtrate, the liquid evaporated down and made up to 10 cc. in a marked test-tube. If the solution has stood with the 5 % acid during the determination, the tube is plugged with cotton wool and autoclaved at 130° for 3 hours. If the process has been carried through without delay, however, the liquid is merely boiled. It is then cooled in ice, and 3 cc. of phosphotungstic acid solution (5 g. sulphuric acid to 20 g. phosphotungstic acid per 100 cc.) are added from a pipette drop by drop, with shaking, as soon as cold. After standing in ice overnight, the precipitate is centrifuged in a 15 cc. urine tube and washed four or five times with a total of 10 cc. phosphotungstic acid wash-liquid (5 g. sulphuric acid to 2·5 g. phosphotungstic acid per 100 cc.), the hard lump of precipitate being stirred up with a glass rod at each washing. After centrifuging for about 10 minutes each time, the liquid can be decanted accurately.

Finally, the precipitate is dissolved out of the cup with 2 cc. of 10 % NaOH, the precipitating tube being rinsed with a few drops of the same, and the nitrogen determined as described above.

Non-basic nitrogen. The combined solution and wash-liquid from above are made up to 50 cc., and the nitrogen estimated in two 10 cc. portions, since to incinerate the entire 4 mg. of nitrogen would be inadvisable. The two portions should agree within 0·1 cc. $N/70$ acid, as otherwise the error ($\times$ 5) is large.

Total nitrogen. This is determined on 2 cc. portions of the hydrolysate, the ratio of the 2 cc. (Ostwald) pipette to the 10 cc. pipette being carefully determined.

The accuracy of the incineration process was carefully checked, using quantities of pure tyrosine weighed out on the microbalance. It was found that up to 3 mg. nitrogen were incinerated and distilled without loss, while the reagent error from various blank determinations was of the order of 0·11 cc. of $N/70$ acid. This was duly allowed for. Parnas and Wagner's distillation apparatus is exceedingly convenient and accurate and was used throughout.

Some of the results obtained by this method are given below. As a general rule the amide nitrogen is higher, and the diamino-nitrogen lower, than given by the macro-method. The latter result is due to the presence of less salt at precipitation, and to more perfect washing on the centrifuge than is possible on a filter.

The gliadin was prepared from wheat flour, and the edestin from hemp seeds according to standard methods. The figures given for gelatin refer to ordinary gelatin in the case of Van Slyke and Hausmann, but to Coignet's Gold Label, purified by electrodialysis and flocculation, in the case of this method.

The entire determination can be carried through in about 3 days, which is roughly half the time occupied by the macro-method.

Protein	Author	Amide-N %	Humin-N %	Diamino-N %	Mono- & non-amino-N %	Total %
Gelatin	Hausmann [1899]	1·6	—	35·8	62·5	99·9
	Van Slyke [1911]	2·2	0·1*	25·5	71·2	99·0
	Knaggs [1923]	—	—	20·0	—	—
	Micro-method	2·6	0·0	17·6	79·6	99·8
Edestin	Van Slyke [1911]	10·0	2·0*	36·7	50·7	99·4
(hemp)	Osborne and Harris [1903]	10·1	0·7†	31·6	57·7	100·1
	Micro-method	12·1	0·3	28·8	58·7	100·0
Gliadin	Van Slyke [1911]	24·5	0·4*	6·2	68·9	100·0
(wheat)	Osborne and Harris [1903]	25·5	0·9†	11·7	61·7	99·8
	Micro-method	26·3	0·1	5·4	68·3	100·1

* Nitrogen in CaO precipitate. † Nitrogen in MgO precipitate.

Summary.

1. The various sources of error in the determination of the Hausmann numbers of the proteins are described.

2. The presence of an uncontrolled amount of salt is shown to be an important factor in determining the composition of the precipitate of the basic fraction.

3. A convenient micro-method for the determination of the Hausmann numbers is described, avoiding the various errors.

The above work was carried out at the suggestion and under the direction of Professor S. B. Schryver.

The author desires to express his thanks to the Department of Scientific and Industrial Research for a grant which enabled him to carry out this work.

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CLII. CONTRIBUTIONS TO THE STUDY OF BRAIN METABOLISM.

III. CARBOHYDRATE METABOLISM RELATIONSHIP OF GLYCOGEN AND LACTIC ACID.

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(*Received October 16th, 1926.*)

In the course of some previous experiments dealing with the carbohydrate metabolism of the brain [Holmes and Holmes, 1925, 1, 2], we observed that the lactic acid content of rabbits' brains showed little or no increase when the tissue was chopped and allowed to stand at room temperature, or was incubated, under anaerobic conditions. This appeared to be the case at p_H 7·6, 8·2, 8·4 and 9·0. In similar circumstances, tissues such as muscle or liver might be expected to show a lactic acid production substantially above the "resting" value, with a corresponding diminution in lactic acid precursor. We further observed that there was no diminution in the "soluble carbohydrate" of brain after the organ had been allowed to stand for 24 hours at room temperature. Since brain tissue readily forms lactic acid from added glucose in these conditions, we deduced that the substances estimated in the extracts did not include glucose. We were subsequently able to show [1926] that the reducing substances which we estimated were largely, if not wholly, creatine and creatinine.

While our experiments were still in progress, Takahashi and Asher [1925] published a paper dealing with the glycogen and "übrigen Kohlenhydrate" content of various tissues, including brain. Since their technique for "soluble carbohydrate" was essentially similar to ours, we have no doubt that the substances (in the brain) which they estimated under that heading were chiefly creatine and creatinine. They found that the glycogen content of the brain, unlike that of other tissues, was not influenced by the treatment to which they subjected their animals, unless the treatment was such as to produce convulsions, in which case the glycogen content diminished. This point is referred to later in this paper. It may here be remarked that, while Takahashi and Asher claim that insulin convulsions markedly reduce the brain glycogen, we found that some of our lowest figures for brain lactic acid were obtained from animals which had convulsed as a result of insulin injection [1925, 2], which is hardly in harmony with their findings.

In view of the criticism which has recently been directed against the application of Pflüger's method for the determination of glycogen to small quantities of tissue [Evans, 1925], we feel it desirable to describe our technique in detail.

As pointed out by Winterstein and Hirschberg [1925], Pflüger's method cannot be applied safely to fresh brain tissue, because the cerebrosides will, partially at least, escape destruction by the potash, and will therefore give rise to galactose in the final hydrolysis and thus vitiate the subsequent estimation of reducing substance. In our experiments the cerebrosides were therefore removed, as suggested by Winterstein, by extraction with alcohol and chloroform. In detail, therefore, our procedure was as follows. The rabbit was killed by a blow on the back of the neck. The brain was removed, freed from membranes and adherent blood clot, and either frozen at once (for "resting" values), or subjected to any desired treatment. The frozen brains were sampled, weighed, and immersed (while still very cold) in 95 % alcohol. To brains that had been in Ringer's solution, three volumes of alcohol were added, and the whole allowed to stand overnight. The tissue, and any precipitate present, was then filtered off through a Jena glass filter (mesh "5–7" or "< 7" was found suitable). If a lactic acid estimation was required, the necessary further extractions were then carried out, the tissue and alcoholic extract being separated by means of the filter; otherwise, the fluid was discarded. The tissue was transferred to a beaker and boiled five times with alcohol and twice with chloroform, being filtered after each extraction through the same filter. The inside of the beaker was carefully scraped and the scrapings transferred to the filter with a little hot chloroform. Finally, the solid was washed twice in the filter with hot alcohol. This treatment removed all substances soluble in hot alcohol or in chloroform.

The powdery mass was next transferred to a 300 cc. Kjeldahl flask. 10 cc. of 60 % potash (Kahlbaum), with which beaker and filter had been rinsed, were added. A condenser was fitted, and the flask heated for 3 hours over a microburner, so adjusted that the potash was a little below its boiling point.

After 3 hours, the contents of the flask were transferred to a 100 cc. beaker: 20 cc. of water, 60 cc. of 95 % alcohol, and a pinch of sodium chloride were added, and the beaker was left overnight. Next day, the precipitate was centrifuged down, washed, taken up in boiling water, filtered, and hydrolysed according to Pflüger's method. The reducing substance was estimated by the method of Hagedorn and Jensen [1923]. Usually 5 cc. of fluid gave convenient readings.

In view of the criticism of Evans [1925] we carried out some experiments to determine whether small quantities of glycogen were precipitated from the comparatively large bulk of fluid (90 cc.) which we used. To a mixture of 10 cc. of 60 % potash, 20 cc. of water, 60 cc. of 95 % alcohol, and a little sodium chloride, known amounts of Kahlbaum's glycogen were added. The subsequent procedure was as described above. Table I shows the results of these experiments.

Table I.

Glycogen added (mg.)	4	3	2	1
„ recovered (mg.)	3·91	3·01	2·15	1·20

As an additional control, a rabbit was killed, and the brain frozen and sampled. Two portions of 4 g. each were weighed out, and glycogen was estimated in each portion, 0·6 mg. of Kahlbaum's glycogen being added to portion A just before the digestion with potash. The figures obtained were as follows:

Portion A	0·882 mg. glycogen
Portion B	0·308 mg. „
Glycogen recovered ...	0·574 mg.
Glycogen added	0·600 mg.

To test out the method, and to endeavour to determine the range of individual variation likely to be met with in normal rabbits, a series of duplicate estimations was carried out on the brains of stock animals. Table II shows "resting" values expressed as mg. of glycogen per 100 g. of fresh brain.

Table II.

Glycogen (mg. %)	A	28·6	41·1	74·8	15·0	14·2	19·6	28·1	43·3
	B	28·9	39·8	70·4	16·9	15·0	15·5	28·7	44·6

It is evident from these figures that, whilst the glycogen content of mammalian brain is very small (a fact noted by previous workers), the variations among normal rabbits, chosen at random from stock, are so large that one cannot regard as valid any conclusions that are based on differences observed between different animals subjected to varying treatment. We were, therefore, compelled to conduct all our experiments on different portions of the same brain.

Table III.

			Resting	Stood or incubated	
No.	Description	p_H	Glycogen mg. %		Remarks
1	Rabbit, normal	9·0	16·22	13·8	Incubated 3 hours at 37°, KCN *M*/500
2	Rabbit, normal	9·0	16·01	12·3	Incubated 3 hours at 37°, KCN *M*/500
3	Rabbit, normal	8·4	28·38	24·38	Stood 24 hours at room temperature, KCN *M*/1000
4	Rabbit, normal	8·2	24·73	22·0	Stood 24 hours at room temperature, KCN *M*/500
5	Rabbit, normal	8·2	13·33	14·92	Incubated 3 hours in nitrogen, no KCN
6	Cat, depancreatised	7·8	43·5	34·7	Stood 24 hours at room temperature, KCN *M*/1000
7	Cat, normal	7·8	26·5	31·1	Stood 24 hours at room temperature, KCN *M*/1000
8	Cat, depancreatised	7·8	31·5	26·0	Stood 24 hours at room temperature, KCN *M*/500
9	Cat, normal	7·8	33·7	25·2	Stood 24 hours at room temperature, KCN *M*/500

In Table III are shown the figures obtained by determining the "resting" value for one-half of a brain, and estimating the other half after it had stood in phosphate Ringer's solution at room temperature, or been incubated under anaerobic conditions.

A few values are included which were obtained in the course of some other work on normal and depancreatised cats.

In Exps. 1 and 2, the "resting" value was obtained from tissue which was frozen and kept in cyanide Ringer's solution at $-2°$, while the companion half was kept for the same period in the incubator. This procedure was planned to eliminate any error that might be due to small amounts of glycogen which had dissolved in the Ringer's solution and stuck to the sides of the beaker after precipitation, thus introducing a source of error into the estimation of the incubated half which was absent from that of the "resting" half. Apparently the precaution was unnecessary. In Exp. 5 the tissue was placed, with 10 cc. of Ringer's solution, in a vacuum tube. The tube was evacuated, filled with O_2-free nitrogen, re-evacuated, and again filled with nitrogen. It was thought that by this means all possibility of the conditions being incompletely anaerobic would be avoided.

Table IV shows some "resting" and maximum values obtained for brain lactic acid by Meyerhof's method [1920, 1]. Except in the case of No. 5—which is the same experiment as No. 5 in Table III—the maximum values were all obtained by allowing the tissue to stand, chopped, for 24 hours at room temperature in cyanide-phosphate Ringer's solution. The figures for Exps. 1–4 were, as a matter of fact, obtained before any glycogen estimations were attempted.

Table IV.

Lactic acid in mg. per 100 g. moist brain

No.	Resting	Stood or incubated	p_H	Remarks
1	73·61	86·18	8·4	Stood 24 hours at room temperature in 10 cc. Ringer, KCN $M/1000$
2	116	107·6	9·0	Stood 24 hours at room temperature in 10 cc. Ringer, KCN $M/1000$
3	100·2	106·5	9·0	Stood 24 hours at room temperature in 10 cc. Ringer, KCN $M/1000$
4	124	123	9·0	Stood 24 hours at room temperature in 10 cc. Ringer, KCN $M/1000$
5	123·3	129·5	8·2	Incubated 3 hours at 37° in Ringer in N_2

In most cases, though not in all—and No. 5 is one of the two exceptions—there is a fall in the glycogen on standing. This fall is extremely small in actual amount, though slightly more imposing when calculated as a percentage, owing to the very low "resting" value. Three of the lactic acid values show a slight rise, two a slight fall.

In this respect the metabolism of the brain is evidently quite different from that of muscle or liver. In the case of the lactic acid values the rise is not constant, and, when it occurs, is so small as to approach the limits of experi-

mental error. Though the fall in glycogen seems to be of more regular occurrence, it too is very small. We are, indeed, disposed to regard it with very great reserve, believing that it is possibly due to some hitherto undiscovered flaw in the technique. It may be mentioned here that one of us (B. E. H.) has observed that there is, apparently, but very little fall in the glycogen content of tumour tissue in circumstances similar to those here described.

We carried out a series of experiments to observe the effect on the brain glycogen of incubated chopped brain tissue under aerobic conditions. Meyerhof [1920, 2] has demonstrated the synthesis of glycogen and the simultaneous disappearance of lactic acid in whole amphibian muscle under aerobic conditions. Naturally, it would be unsafe to assume that such a synthesis would occur in our chopped brain preparations, even supposing that it took place in the brain of the living animal, since Meyerhof found it necessary to employ intact muscle, and other workers to employ carefully sliced pieces of tissue.

Winterstein and Hirschberg [1925], however, reported a fall in the glycogen content of frogs' central nervous system when the tissue was kept, unstimulated, in oxygenated sodium chloride solution. Whilst under anaerobic conditions the glycogen content remained unchanged, he observed it to rise when the central nervous system was electrically stimulated in circumstances ensuring an adequate oxygen supply. Our values are arranged in Table V. The brains were halved and the "resting" value was determined on one half, whilst the other was chopped, placed in Ringer's solution in a wide, closed bottle, and shaken in a water-bath at 37° sufficiently vigorously to ensure thorough aeration. The fall in glycogen content observed is not materially greater than that seen under anaerobic conditions.

Table V.

		Glycogen (mg. %)		
No.	p_H	Resting	Shaken 3 hours	Description
1	8·0	40·55	33·8	Rabbit
2	7·8	31·7	28·4	Rabbit
3	7·8	43·5	33·1	Cat (depancreatised)
4	7·8	31·5	36·5	Cat (depancreatised)

A few experiments were done to demonstrate the fall in lactic acid, which, under aerobic conditions, occurs readily in a preparation of chopped brain.

Table VI.

	Lactic acid (mg. %)		
No.	Resting	Final	Remarks
1	91·3	15·4	Rabbit, normal. Shaken, as in Table V
2	97·4	36·5	Rabbit, normal. O_2 bubbled through Ringer at 37°
3	104	41	Cat, normal. Shaken, as in Table V

Provided that all cerebrosides are previously extracted from the tissue, the use of Pflüger's method in itself offers some guarantee that the material estimated really is glycogen. In addition, Takahashi [1925], using a different

method, claims to have isolated glycogen from his extracts. We felt, however, that it would be advisable to attempt an identification of our material.

We accumulated, therefore, all the hydrolysed residues left over from the rabbit brain estimations. Finally, about 1300 cc. of fluid were collected, containing some 40 mg. of reducing substance, reckoned as glucose. This was neutralised, concentrated, and treated in the ordinary way with phenylhydrazine. Crystals were obtained having the appearance, under the microscope, of those of glucosazone. Unfortunately they were lost during an attempt at recrystallisation, so we have no data as to melting-point. More material is being accumulated.

Athanasiu [1899] observed glycogen in the central nervous system of the frog, and gave a value of 70 mg. %. Schörndorff [1905] estimated glycogen in dog's brain, finding values of from 190–260 mg. %. From the text it is clear that part of his reducing substance arose from the hydrolysis of cerebrosides. Takahashi [1925] and Takahashi and Asher [1925] report data relating to rat, rabbit, and human brains. They find that there is no fall in brain glycogen after insulin administration (provided convulsions are avoided), peptone feeding, heavy muscular work, thyroid or phloridzin administration, although they find marked diminution of glycogen in other organs in these circumstances. If, however, convulsions have occurred, they believe that the brain glycogen does diminish. We are unable to accept their conclusions for rabbits, because they report only a few experiments, and we believe that the differences which they find might easily be covered by the very wide range of normal variation. Uchida [1925] describes experiments showing a fall in glycogen content of the brains of rats which have been exposed to the action of anaesthetics, as compared with those of normal animals. He uses a method based on a technique used by Rona and Von Eweyk [1924] for isolating pure glycogen from tissues, which, apparently, was not intended to be quantitative. The glycogen values which we have obtained for anaesthetised rabbits fall well within the very wide range of normal values; possibly rats' brains show a less variable glycogen content. Kobori [1926] states that the glycogen content of doves' brains rises markedly after convulsions produced by methylguanidine. He gives some figures which, he claims, support Takahashi's contention that the brain glycogen is diminished by insulin convulsions. Again, so far as rabbits are concerned, the figures fall almost within the range of variation observed by us in normals.

We realised that it is possible that the low values which we, in common with other workers, find for the glycogen content of the brain, may be due, not to a slow or inadequate breakdown mechanism, but to an extremely rapid one. It is conceivable that by no technique hitherto described is it possible to obtain a true "resting" value, since the methods employed for killing animals all involve either the use of an anaesthetic, or a violent, if momentary, stimulus to the central nervous system, which might result in a large glycogen breakdown before the tissue can be fixed. Such an explanation

would not, however, account for the absence of further significant breakdown *in vitro* at alkaline p_H, to explain which it would be necessary to postulate some further mechanism which was operative only in the living animal. We are, moreover, endeavouring to employ other methods of obtaining a true "resting" figure for glycogen, and, although our experiments are incomplete, we have so far failed to find any indication of a higher figure.

In special circumstances, we have found a slight increase above the "resting" value in the lactic acid figure for rabbits' and cats' brains under anaerobic conditions. Gesell [1925] reports a similar rise in the figure for dogs' brains. In our experience, this has never been accompanied by a corresponding fall in glycogen.

Loebel [1925] was unable to demonstrate increased lactic acid production when glycogen was added to brain preparations. This observation, perhaps, lacks very much significance from our point of view, since glycogen added to tissue preparations frequently fails to behave in a manner comparable to that of the glycogen already contained in the tissue, on account, presumably, of its physical properties.

We can find no evidence that glycogen behaves as a lactic acid precursor in the mammalian brain. It has been shown that brain tissue forms lactic acid with great rapidity from glucose [Warburg, Posener and Negelein, 1924; Holmes and Holmes, 1925, 1], and we were also able to show [1925, 2] that, after insulin, the lactic acid content of rabbits' brains runs, roughly, parallel to the blood sugar at the time of death. It seems to us, therefore, that it is quite possible that glycogen plays a comparatively unimportant (or at least quite obscure) part in the carbohydrate metabolism of mammalian brain, and that the organ is dependent for lactic acid precursor directly on the glucose supplied to it by the blood.

SUMMARY.

1. The glycogen content of rabbits' brains is small, and very variable.

2. The lactic acid content of rabbits' brains shows no appreciable rise, nor does the glycogen content show any significant fall, when the chopped tissue is kept at room temperature, or incubated under anaerobic conditions at alkaline p_H.

3. Under aerobic conditions, lactic acid rapidly disappears from chopped brain, but the glycogen suffers no significant change.

4. It is suggested that the brain depends upon the blood sugar, rather than on any substance which it stores itself, for lactic acid precursor.

One of us (B. E. H.) is in receipt of a grant from the Medical Research Council.

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CLIII. THE ESTIMATION OF PHOSPHORUS IN BLOOD.

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THE results obtained in the colorimetric estimation of phosphorus are known to be affected by a number of factors [Fiske and Subbarrow, 1925] and various workers have applied more direct methods to the small quantities of phosphorus present in blood. Using Neumann's method [1903] Iversen [1920] was successful in estimating 0·015 mg. of phosphorus with an error of ± 0·003 mg. Stewart and Archibald in a later paper [1925] estimated 0·05 mg. with an error of ± 0·0015 mg.

It has been found that the introduction of various modifications simplifies the method and allows 0·013 mg. to be estimated with an error of ± 0·0004 mg. The precipitate is washed with the ammonium nitrate solution described below and the washing is carried out in small centrifuge tubes similar to those described by Trevan and Bainbridge [1926]. The micrometer syringe described by Trevan [1925] has been used for the titration.

Titration readings.

After 1 washing	0·591	cc. of	$N/10$ HCl
,, 2 washings	0·544	,,	,,
,, 3 washings	0·541	,,	,,
,, 4 washings	0·541	,,	,,
,, 5 washings	0·542	,,	,,

To remove the ammonia that is formed when ammonium phosphomolybdate is dissolved in soda it is necessary to boil the solution until its bulk is considerably reduced [Iversen, 1920] but more consistent results have been obtained by titrating in the presence of the ammonia.

To determine the optimum p_H for the end-point, the titration curves of these solutions have been plotted with indicators. They have also been plotted by Dr Trevan with a glass electrode and similar results have been obtained. It will be seen that the best end-point is about 7·4 (see Fig. 1). This is an unexpected result because the phosphate solutions themselves buffer well in this region.

The titration in a CO_2-free atmosphere involves a slight modification of the usual technique for the micrometer syringe. A long glass needle drawn out

fine is made to dip below the surface of the liquid which is kept stirred by a constant stream of bubbles of CO_2-free air. It has been found convenient to pump the air with a 2-foot Kekulé pump [Barr, 1924]. The air then passes through a jar of soda lime (which is renewed each morning) to the titration tube. The bubbles cause a certain amount of splashing on the sides of the tube. The error so introduced is less than 1 % of the total titration and the liquid soon runs down again. When the titration is nearly completed the tube may be taken out and tilted to ensure that no unneutralised acid remains on the sides of the tube. Paradoxically, splashing may often be abolished by increasing the rate of bubbling.

Preparation of the blood.

"Inorganic phosphate" is conveniently measured in whole blood. A quantity of blood is measured straight into trichloroacetic acid in accordance with the advice of Fiske and Subbarrow [1925]. It was found that a concentration of 3·3 % trichloroacetic acid in the final solution was sufficient for the precipitation of proteins if it was left for 2 hours before centrifuging. 0·5 cc. of blood is measured into 2·5 cc. of 4 % trichloroacetic acid and 2 cc. of the supernatant fluid are taken for phosphorus determination.

Solutions containing "total acid-soluble phosphate" have been prepared by the method of Fiske and Subbarrow [1925] and solutions for "ether-soluble phosphate" by the method of Randles and Knudson [1922].

ESTIMATION OF PHOSPHORUS.

Solutions required.

(1) Sulphate-molybdate reagent.

50 mg. of ammonium sulphate are dissolved in 500 cc. of nitric acid of specific gravity 1·36 in a litre flask. 150 g. of powdered ammonium molybdate are treated with 400 cc. of boiling water in a porcelain dish and stirred until solution is complete. The solution is rinsed into a flask with a little water, cooled to the temperature of the room, and poured in a thin stream with stirring into the nitric acid solution of ammonium sulphate. The resultant liquid is diluted to 1 litre, allowed to stand for 2 days, filtered and kept in a well stoppered bottle in a dark cool place [Pregl, 1924].

(2) 30 cc. of sulphuric acid (S.G. 1·84) are poured into 1 litre of nitric acid (S.G. 1·19–1·21). The latter acid is obtained by mixing 357 cc. of nitric acid of S.G. 1·4 with 500 cc. of water.

(3) 2 % solution of ammonium nitrate in distilled water containing CO_2 with the addition of one drop of 2 % nitric acid per 100 cc. so that the p_H is about 4.

(4) Normal HCl and NaOH (CO_2-free).

2 cc. of the unknown phosphate solution are measured into a small hard glass test-tube which is drawn out to a point at one end. 0·25 cc. of sulphuric-nitric acid is added with a syringe and the test-tube is heated to boiling point in a water-bath. It is then removed and 2 cc. of the molybdate reagent are

added. This must be measured as accurately as the phosphate solution, since it was found that a 12 % error in this measurement might make a 10 % error in the titration.

It is allowed to stand for a minute or two because it was found not to pack well when centrifuged at once though the titration values were correct within 1 %. The tube is centrifuged at about 3000 revolutions per minute for 15 minutes at a radius of 10 cm. If it has previously been cleaned by boiling in potassium dichromate and sulphuric acid the precipitate packs neatly in the bottom of the tube. The liquid is poured off carefully and the tube inverted and its mouth dried with blotting paper. 5 cc. of the ammonium nitrate solution is then added from a pipette, and two tubes may be centrifuged for 5 minutes without rebalancing. Two washings with this solution are sufficient. The delivery tube of the CO_2-free air is placed in the test-tube and the air is allowed to run for a minute to drive out the CO_2. A standard quantity (about 0·5 cc.) of CO_2-free $N/10$ soda is measured with a micrometer syringe and added through a long tube and the precipitate rapidly dissolves in this. A drop of phenol red (0·02 %) is added, and the soda titrated without undue delay to p_H 7·4. The difference between this reading and a standard reading taken without any precipitate gives the volume of HCl equivalent to the phosphorus. By titrating a known solution of KH_2PO_4, it was found that 253 cc. of $N/10$ HCl corresponded to 31 mg. of P.

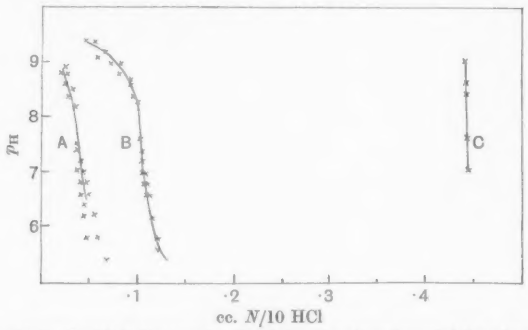

Fig. 1. Curves obtained with indicators by dissolving the phosphomolybdate from 0·0415 mg. of P in the same quantity (about 0·44 cc.) of $N/10$ soda and titrating with $N/10$ HCl. Curve *A*, after boiling off the ammonia. Curve *B*, without boiling off the ammonia. The points on each of these curves were obtained in two separate titrations. Curve *C*, standard titration without any phosphomolybdate. The best end-point is at p_H 7·4.

Results.

cc. of N acid calculated to be equivalent to 31 mg. of P.

At p_H 7·4 Neumann's formula would give	28·5
Pregl's formula would give	*ca.* 29·5
Stewart and Archibald [1925]	22·35
Titrated after boiling off the ammonia	*ca.* 30·4
Titrated without boiling off the ammonia	25·3

0·5 cc. of a batch of normal horse serum gave readings:

0·0124 mg.
0·0126 mg.
0·0131 mg.
0·0132 mg.

Average 0·0128 mg. or 2·56 mg. per 100 cc.
Maximum deviation 0·0004 mg.

2 cc. gave readings:

0·0518 mg.
0·0522 mg.

Average 0·0520 mg. or 2·6 mg. per 100 cc.
Maximum deviation 0·0002 mg.

SUMMARY.

The "inorganic phosphate" in 0·5 cc. of blood may be estimated by dissolving the precipitate of phosphomolybdate in $N/10$ soda and titrating the excess soda with $N/10$ HCl by means of a micrometer syringe.

I wish to thank Dr Trevan for his encouragement and advice.

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CLIV. THE PREPARATION AND PHYSICO-CHEMICAL PROPERTIES OF SERICIN.

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It is usually accepted that silk is in the main composed of two proteins, designated as sericin and fibroin. A fairly large number of investigations of the chemical composition of these proteins have been carried out, though their physico-chemical properties have been little studied. As silk is not only a very important material in the textile industry but also a substance of biological interest, it is desirable to obtain information concerning these properties. The present paper is chiefly concerned with the study of the physico-chemical properties of sericin.

I. Preparation of sericin.

Several methods of preparing sericin have been proposed, but none seems to be satisfactory. Cramer [1865] extracted sericin with boiling water and precipitated it with lead acetate. The precipitate was decomposed by hydrogen sulphide and the protein separated by alcohol.

Bondi [1902] has described two methods. In the first the sericin was extracted by boiling the cocoons in water for 1 hour. This extraction was repeated three times, the water each time being renewed. All the extracts were collected and evaporated to dryness. The residue was powdered, treated with water repeatedly to free it from impurities soluble in water, then dissolved in alkali and precipitated by alcohol. The second method differs from the previous one in precipitating the sericin in the aqueous extract by acetic acid and then treating the precipitate thus obtained with alcohol and ether to reduce it to powder. Fischer [1908], Türk [1920], Kondo [1921] and several other investigators have extracted sericin from the cocoons in an autoclave at a temperature of 118° or more, and precipitated the sericin with alcohol of a concentration not less than 50 %.

Quite recently Shelton and Johnson [1925], after a careful survey of the previous methods, concluded that the best way to extract is at 105° in the autoclave. They asserted also that there are two kinds of protein, one soluble in water and the other insoluble.

Experimental.

1. *Influence of heating at various temperatures on the precipitability of sericin at a certain acidity.*

Since there is a wide divergence of opinion among many investigators with regard to the extraction-temperature, it is necessary to decide first of all the most suitable temperature in order to get the greatest yield and also to extract the protein in its natural state.

20 g. of cocoon, freed from the chrysalides, were soaked in water for a few hours and then extracted with 500 cc. of distilled water at 100° and 105° in an autoclave for 1 hour. The extractions were repeated three times, the water each time being renewed. The nitrogen content in each extract was determined and compared with that of the filtrate which was obtained by filtering off the precipitate caused by the addition of acetate mixture of p_H 3·8, which seems to give the most copious precipitation, as shown later. The difference of the two values gives the amount of protein precipitated at the above acidity. In the same way the amount of the protein precipitable by 25 % alcohol in the filtrate was calculated. The results are given in Table I (where quantities are expressed in milligrams).

Table I.

Extraction-temperature	Number of extraction	N in 100 cc. of extract	N precipitated by acetate mixture	N precipitated by 25 % alcohol
105°	1	146·4	9·4	58·1
	2	16·8	2·4	6·7
	3	2·0	0	0·8
100°	1	68·3	39·2	21·0
	2	66·0	54·0	1·5
	3	15·3	13·8	0·2

The table clearly shows that, though extraction at 105° in the autoclave was much more effective than at 100°, it induced an alteration in the nature of the protein, *i.e.* the portion precipitated by the acetate mixture becomes very much reduced and that by alcohol increased. To verify this fact the following experiment was carried out. The aqueous extract at 100°, which contained 52·8 mg. nitrogen in 100 cc., of which 32·1 mg. belonged to the portion precipitated by the acetate mixture, was heated in the autoclave for 1 hour at a high temperature, and nitrogen determinations were made on the filtrate in the same way as in the foregoing experiment. The results are given in Table II (quantities of nitrogen are expressed in milligrams).

We see from this experiment that, as the temperature was raised, the amount of the protein precipitated by the acetate mixture decreased and a higher concentration of alcohol was required for the precipitation of the protein in the filtrate. It may be concluded, therefore, that heating at a high temperature causes some change in or decomposition of the protein molecule (or protein aggregate).

Table II.

The original solution contained 52·8 mg. N in 100 cc.

Temperature of autoclave	N precipitated by acetate mixture	N precipitated by alcohol of the final concentration			
		25 %	40 %	50 %	75 %
100°	32·1	20·0	20·4	20·4	20·4
105°	12·4	36·1	37·5	38·0	38·0
110°	10·2	29·9	40·6	41·0	41·0
112°	9·4	29·9	40·0	41·0	41·0
125°	7·1	26·8	31·1	36·4	44·0
130°	6·2	7·0	16·9	25·2	40·2
140°	6·2	0·6	6·6	10·2	14·6

2. *Influence of heating on the chemical character of sericin.*

The typical properties of the proteins are due to the presence of the amino-acid groups which build up the protein molecule. In the case of sericin, whether the alteration of its physical properties by heating is to be ascribed merely to the splitting of the carbamino-linkage, or is accompanied by the destruction of certain amino-acids is an interesting question. The writer does not touch this problem here, but one fact must be emphasised in connection with the method of preparation, namely, that the Hopkins-Cole glyoxylic acid reaction for tryptophan is positive with the protein precipitated by the acetate mixture, but negative with the protein precipitated by alcohol.

3. *The optimal H ion concentration for the precipitation of sericin.*

The aqueous extract was rendered acid by adding an acetate mixture of varying H ion concentration and the amount of the precipitate observed after the lapse of 1 hour. In the same way the precipitate produced by alcohol in the filtrate from the above precipitate was noted. The results are indicated in the following table:

Table III.

p_H of the acetate mixture	The amount of the first precipitate	The amount precipitated by alcohol
3·2	+ +	+ +
3·4	+ +	+ +
3·6	+ + +	+ + +
3·8	+ + + +	+ + +
4·0	+ +	+ +
4·2	+ +	−
4·4	−	−

After standing overnight there was no difference between the range from p_H 3·4–4·0.

4. *Discussion of the methods of various investigators.*

On the basis of the above experiments it is now apparent that the method of using an autoclave in the extraction of sericin is least to be recommended. Especially should such a temperature as 118°, introduced by Fischer, be avoided. On the other hand, the method adopted by Anderlini [1887, 1888],

namely, to extract in water at 50–60° for 25 days, is very tedious. By Cramer's method we can get a mixture of two proteins, one precipitable by the acetate mixture and the other not. Bondi's first method also gives such a mixture. Shelton and Johnson's second method of fractionating by means of ammonium sulphate is good, but Bondi's second method seems to be the best, which the writer has modified as follows.

5. *The writer's method of preparing sericin.*

The cocoon used was the Italian yellow Lombardi.

100 g. of cocoon, carefully freed from the chrysalides, were soaked in water for 6 hours (excessive soaking without antiseptics must be avoided), washed thoroughly with water several times, and extracted by boiling for 90 minutes in 20–30 volumes of distilled water. The extraction was repeated three times. All the extracts were collected and filtered through a thick paper mat in a Büchner funnel. A clear yellow solution was obtained. To this solution *N* acetic acid was added drop by drop with good stirring until the p_H of the whole solution attained about 3·9–3·8, about 100 cc. of the acid being necessary. Soon a thick yellow precipitate appeared and settled down to the bottom. After standing a few hours, the supernatant fluid was decanted and the precipitate washed several times with water. The decanted solution and the wash-waters were collected and kept for the isolation of the water-soluble portion of sericin. The precipitate was transferred to a flask and boiled with a sufficient amount of 25 % alcohol for a few minutes. This process was repeated until the alcohol was colourless. Then the precipitate was brought to the state of a dry powder by means of alcohol and ether. The yield was about 27 g. For further purification this crude sericin was dissolved in dilute ammonia, warming to 60–70°. The solution was filtered through a clean paper mat in a Büchner funnel and the sericin reprecipitated by adding acetic acid. The process was repeated twice, the last precipitation being produced by dilute hydrochloric instead of acetic acid. The precipitate was washed with water until there was no chloride reaction in the wash-water and then reduced to powder by treatment with aqueous alcohol solutions of increasing strength and finally with dry ether. The sericin thus obtained is a somewhat yellowish coloured powder, soluble with difficulty in water, but soluble in alkali and in acid of a certain concentration. Its ash content is 0·173 % and N content 16·04 %.

The solution kept for the isolation of the water-soluble sericin was evaporated under diminished pressure to a thick syrup, which was poured into 25 % alcohol. The greater part of the protein was precipitated and filtered through a hard filter paper. The precipitate was dissolved in distilled water, filtered through the paper mat and, after having been reprecipitated by alcohol, was brought to a state of powder by treatment with alcohol and ether. The substance thus obtained is pure white and easily soluble in water. Its N content is 17·05 %. The writer designates this substance *sericin peptone*. Whether this is solely a decomposition product of the protein precipitable by acetic acid

or a mixture containing also a different kind of protein existing *ab initio* in the cocoon, must be ascertained by further researches.

II. Physico-chemical properties of sericin.

1. *The solubility of sericin.*

The perfectly dried sericin, prepared as above, was very slightly soluble in water; but when boiled with water, it went into solution to a slight extent, perhaps owing to the alkali from the glass. As will be seen later, the sericin is a protein of marked acid character so that it dissociates in water as an acid and becomes soluble. Its solubility, therefore, depends solely upon the degree of dissociation, which is again a function of the acidity of the medium. The following experiments give data concerning its solubility at different H ion concentrations.

0·2 g. portions of the powdered sericin, which passed through a No. 40 sieve, were weighed out into a series of flasks. To each of them 20 cc. portions of 0·2 *M* acetate mixture of varying H ion concentrations, and of the compositions shown in the following table, were added. The flasks were put into a thermostat at 25° and occasionally stirred. After the lapse of 2 hours the contents were filtered and the amount of protein in the filtrates determined by means of Kingslake's nephelometer. The turbidity was produced by bringing the solutions to the acidity of p_H 3·8 (which was shown to be the best for producing precipitation in the previous experiment) and compared with that of a standard solution, which contained 50 mg. of sericin and 0·01 mol NaOH in 100 cc. The results are given in Table IV and Fig. 1.

Table IV. *Solubility of sericin at different acidities.*

Acetic acid 0·2 *M* cc.	Na-acetate 0·2 *M* cc.	p_H (electrometric)	Amount of sericin dissolved in 100 cc. mg.
20	0	2·64	31·1
19·5	0·5	3·16	25·5
19·0	1·0	3·43	11·7
18·5	1·5	3·60	9·6
17·6	2·4	3·86	0
16·4	3·6	4·07	5·8
14·7	5·3	4·27	23·9
12·6	7·4	4·46	31·0
10·6	9·8	4·68	51·8
5·9	14·1	5·07	53·6
2·9	17·1	5·42	60·7

The above table and the accompanying figure (Fig. 1) clearly demonstrate that the solubility of the sericin is a minimum near p_H 3·9 and increases on both sides from this point. Since it is usually assumed that a protein, as an ampholyte, is least soluble at its isoelectric point, we can conclude that this point lies near p_H 3·9 in the case of sericin. Some other physico-chemical properties also lead to the same conclusion.

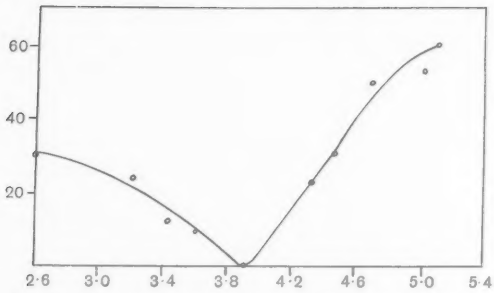

Fig. 1. Milligrams of sericin dissolved (ordinate) in 100 cc. of acetate mixture (p_H abscissae).

2. *Swelling of sericin.*

Procter and Wilson and J. Loeb have developed a theory of the mechanism of swelling upon the basis of Donnan's membrane equilibrium theory. According to them the swelling of the protein must be a minimum at its isoelectric point. This is actually the case with sericin.

To 0·1 g. portions of sericin in a number of test-tubes of uniform size (7 × 50 mm.) 2 cc. portions of the acetate mixtures of different H ion concentrations were added. After being well mixed they were put into a thermostat at 25°, and at the end of 24 hours the height of the swollen sericin was read. The results are illustrated in Fig. 2.

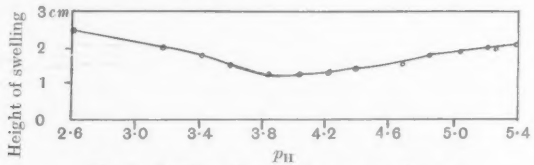

Fig. 2. Swelling of sericin in acetate mixture.

The same experiment was repeated, using HCl and NaOH of varying concentrations, instead of the acetate mixture, the result of which is shown in Fig. 3.

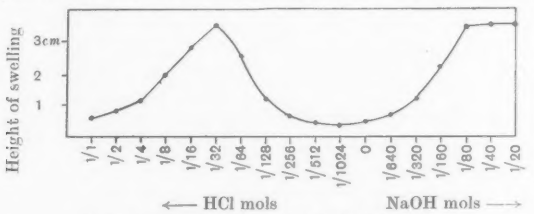

Fig. 3. Swelling of sericin in HCl and NaOH.

The curves show apparently a drop near the isoelectric point. Moreover, it is noticeable that in the region of greater acidity the curve falls again after attaining a maximum at 1/32 *M* HCl. Though the amount of sericin dissolved,

which was determined by the micro-Kjeldahl method of Folin in an aliquot portion of the supernatant clear fluid, increased with the increase of acid as shown in Table V, we can still recognise that the swelling of sericin is retarded at the higher acidities.

Table V. *The amount of sericin dissolved in 2 cc. of various concentrations of HCl.*

The total concentration of sericin = 100 mg.

Conc. of HCl (*M*)	2/1	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
Sericin dissolved (mg.)	10·0	8·9	8·3	6·5	8·0	—	—	—	7·0	3·0	2·4	0

3. *The viscosities of sericin-HCl and sericin-NaOH mixtures.*

To a certain extent the viscosity of a colloidal solution is defined by the magnitude of the micelle. In this sense it must run parallel to the swelling.

0·2 g. portions of powdered sericin were weighed out into a series of test-tubes, to which 20 cc. portions of HCl of concentrations varying from 1/5 to 1/640 *M* were then added. To facilitate the dissolution the tubes were put into a water-bath at 80° for 20 minutes, the contents being stirred at times with a glass rod. After this procedure, one portion of the mixture was filtered through filter paper. Both mixture and filtrate were cooled in running water and transferred to a thermostat at 25°, in which the viscosity was measured at the end of 30 minutes. A viscometer of Ostwald's type was used. The above procedure was followed strictly, because the viscosity varied with the time and the temperature and duration of dissolution. The viscosity was expressed as the ratio t/t_0, where t and t_0 are the times for the flow of the sericin solution and water respectively. In the same solution the H ion concentration and the conductivity were measured. The calculation of the former was carried out by the following formula,

$$p_H = \frac{E - 0{\cdot}2464}{0{\cdot}05911},$$

where E is the E.M.F. observed against the saturated calomel electrode at 25°. No correction was made for liquid potential. Further, the amount of sericin dissolved in the filtrate and the non-precipitable portion in it, when neutralised by adding the same amount of acid or base used to dissolve the protein, were determined and are given in columns 5 and 6 in Tables VI *a* and *b* respectively. The difference of these two values gives the amount of sericin precipitable at the isoelectric point.

Table VI *a*. *The viscosity of the sericin-HCl mixtures.*

HCl added *M*	p_H	Relative viscosity of		The amount of sericin (mg.) dissolved in 100 cc.		
		Mixture	Filtrate	Total	Non-precip.	Precip.
1/5	0·804	1·10	1·10	—	—	—
1/10	1·17	1·32	1·30	0·367	0·145	0·222
1/20	1·43	2·24	2·20	0·700	0·080	0·620
1/40	1·78	1·55	1·51	0·773	0·038	0·735
1/80	2·17	2·72	1·74	0·850	0	0·850
1/160	2·65	2·09	1·65	0·745	0	0·745
1/320	3·22	1·77	1·50	0·667	0	0·667
1/640	3·57	1·08	1·08	0·024	0	0·024

Table VI *b*. *The viscosity of the sericin-NaOH mixtures.*

NaOH added *M*	p_H	Relative viscosity of Mixture	Relative viscosity of Filtrate	The amount of sericin (mg.) dissolved in 100 cc. Total	Non-precip.	Precip.
1/10	12·71	1·19	1·18	1·000	0·330	0·670
1/20	12·33	1·24	1·23	1·000	0·218	0·792
1/40	11·75	1·53	1·53	1·000	0·008	0·992
1/80	10·83	2·27	2·18	1·000	0	1·000
1/160	7·64	3·10	2·90	0·934	0	0·934
1/320	5·78	2·27	1·98	0·840	0	0·840
1/640	5·18	1·45	1·40	0·608	0	0·608
Aq.	4·48	1·07	1·07	—	—	—

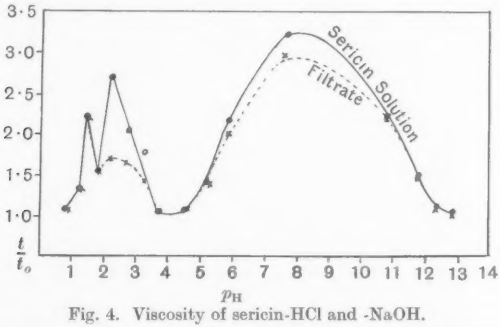

Fig. 4. Viscosity of sericin-HCl and -NaOH.

From the shape of the curve it will be seen that the viscosity is at a minimum near p_H 3·9 and attains a maximum on both sides, as is usually the case with other proteins. One peculiar fact is that there are two maxima on the acid side, the one which occurred at the lower acidity, however, became less marked in the filtrate. This is no doubt caused by the highly swollen micelle. The second maximum is difficult to explain. As a matter of fact, in such a peculiar heterogeneous solution as sericin-HCl, the viscosity is a very complicated thing. Adolf [1923] observed a similar rise of viscosity in the case of globulin-oxalic acid, but her explanation does not appear to be satisfactory.

4. *The amount of HCl or NaOH with which sericin combines.*

This amount was calculated from the values in Table VI *a* by the expression $a - b/\alpha$, where a is the concentration of total HCl added, b that calculated from the p_H of the solution (free HCl) and α the degree of dissociation calculated from the ratio of the equivalent conductivities. A similar calculation holds for NaOH.

Table VII *a*. *The number of mols of HCl combined per* 0·2 *g. sericin in solutions of the given HCl concentrations containing* 1 *g. sericin per* 100 *cc.*

All concentrations are given in mols per litre.

Total HCl (*a*)	Free HCl from p_H p_H	Free HCl from p_H (*b*)	$1/\alpha$	b/α	Combined HCl
0·2011	0·80	$1·57 \times 10^{-1}$	1·095	$17·24 \times 10^{-2}$	$2·87 \times 10^{-2}$
0·1	1·17	$6·74 \times 10^{-2}$	1·059	$7·14 \times 10^{-2}$	$2·86 \times 10^{-2}$
0·05	1·43	$3·76 \times 10^{-2}$	1·035	$3·82 \times 10^{-2}$	$1·18 \times 10^{-2}$
0·025	1·78	$1·67 \times 10^{-2}$	1·025	$1·72 \times 10^{-2}$	$7·86 \times 10^{-3}$
0·0125	2·17	$6·84 \times 10^{-3}$	1·016	$6·95 \times 10^{-3}$	$5·55 \times 10^{-3}$
0·00625	2·65	$2·24 \times 10^{-3}$	1·010	$2·27 \times 10^{-3}$	$3·98 \times 10^{-3}$
0·00313	3·22	$6·00 \times 10^{-4}$	1·000	$6·00 \times 10^{-3}$	$2·53 \times 10^{-3}$
0·00157	3·57	$2·70 \times 10^{-4}$	1·000	$2·70 \times 10^{-4}$	$1·30 \times 10^{-3}$

Table VII *b*. *The number of mols of NaOH combined per* 0·2 *g. sericin in solutions of the given NaOH concentrations containing* 1 *g. sericin per* 100 *cc.*

All concentrations are given in mols per litre.

Total NaOH (a)	p_{OH}	Free NaOH from p_{OH} (b)	$1/a$	b/a	NaOH combined
0·09146	1·29	$5·13 \times 10^{-2}$	1·095	$5·61 \times 10^{-2}$	$3·54 \times 10^{-2}$
0·05	1·74	$1·82 \times 10^{-2}$	1·055	$1·91 \times 10^{-2}$	$3·09 \times 10^{-2}$
0·025	2·35	$4·47 \times 10^{-3}$	1·024	$4·58 \times 10^{-3}$	$2·04 \times 10^{-2}$
0·020	2·51	$3·09 \times 10^{-3}$	1·018	$3·15 \times 10^{-3}$	$1·68 \times 10^{-2}$
0·0125	3·28	$5·31 \times 10^{-4}$	1·000	$5·31 \times 10^{-4}$	$1·20 \times 10^{-2}$
0·01	4·54	$2·88 \times 10^{-5}$	1·000	$2·88 \times 10^{-5}$	$9·72 \times 10^{-3}$
0·00625	6·43	$3·74 \times 10^{-7}$	1·000	$3·74 \times 10^{-7}$	$6·25 \times 10^{-3}$
0·005	7·53	$2·69 \times 10^{-8}$	1·000	$2·69 \times 10^{-8}$	$5·00 \times 10^{-3}$
0·00313	8·29	$5·14 \times 10^{-9}$	1·000	$5·14 \times 10^{-9}$	$3·13 \times 10^{-3}$
0·00157	8·89	$1·29 \times 10^{-9}$	1·000	$1·29 \times 10^{-9}$	$1·57 \times 10^{-3}$

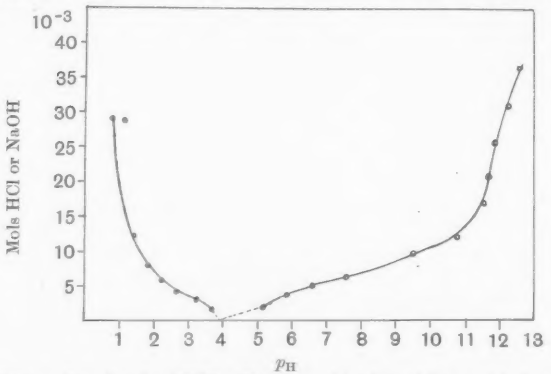

Fig. 5. The number of mols of HCl or NaOH combined per 0·2 g. sericin in solutions of the given p_H values containing 1 g. sericin per 100 cc.

As shown in the above experiment, there seems to exist no saturation limit in the combining capacity of sericin for HCl and NaOH as far as the concentrations of this experiment are concerned. At higher concentrations, namely, beyond p_H 1·43 and p_{OH} 2·35, a sudden increase occurred, perhaps owing to the splitting of the protein molecule. At the lower concentrations both curves show that the minimum of combining capacity lies near p_H 3·9.

5. *The conductivity of the sericin-HCl solutions.*

The measurement of the electrical conductivity was made at 25° as rapidly as possible (1–5 minutes) after that of the viscosity, using a vessel of the Arrhenius type. The specific conductivity due to the sericin-HCl (K_s) was calculated by the expression

$$K_s = K_t - K_f,$$

where K_t = the total specific conductivity, obtained from the experiment.

K_f = the specific conductivity due to free HCl or NaOH calculated by multiplying the concentration of free HCl or NaOH by 0·422 or 0·22, which are the specific conductivities of M HCl or M NaOH solution at 25° respectively.

Table VIII *a*. *The conductivities of sericin-HCl mixtures.*

HCl added (mol)	K_t	HCl from p_H (*b*)	0·422.*b* K_f	K_s
0·1	$3·776 \times 10^{-2}$	$6·74 \times 10^{-2}$	$2·846 \times 10^{-2}$	$9·30 \times 10^{-3}$
0·05	$1·866 \times 10^{-2}$	$3·76 \times 10^{-2}$	$1·587 \times 10^{-2}$	$2·76 \times 10^{-3}$
0·025	$7·615 \times 10^{-3}$	$1·67 \times 10^{-2}$	$7·052 \times 10^{-3}$	$5·36 \times 10^{-4}$
0·0125	$2·957 \times 10^{-3}$	$6·84 \times 10^{-3}$	$2·887 \times 10^{-3}$	$8·00 \times 10^{-5}$
0·00625	$1·056 \times 10^{-3}$	$2·24 \times 10^{-3}$	$9·457 \times 10^{-4}$	$1·11 \times 10^{-4}$
0·00313	$3·455 \times 10^{-4}$	$6·00 \times 10^{-4}$	$2·531 \times 10^{-4}$	$9·24 \times 10^{-5}$
0·00157	$2·249 \times 10^{-4}$	$2·70 \times 10^{-4}$	$1·156 \times 10^{-4}$	$1·09 \times 10^{-4}$

Table VIII *b*. *The conductivities of sericin-NaOH solutions.*

NaOH added (mol)	K_t	NaOH from p_{OH} (*b*)	0·22.*b* K_f	K_s
0·09146	$1·623 \times 10^{-2}$	$5·13 \times 10^{-2}$	$1·128 \times 10^{-2}$	$4·950 \times 10^{-3}$
0·05	$8·121 \times 10^{-3}$	$1·82 \times 10^{-2}$	$4·004 \times 10^{-3}$	$4·117 \times 10^{-3}$
0·025	$3·027 \times 10^{-3}$	$4·47 \times 10^{-3}$	$9·837 \times 10^{-4}$	$2·043 \times 10^{-3}$
0·0125	$8·135 \times 10^{-4}$	$5·31 \times 10^{-4}$	$1·195 \times 10^{-4}$	$6·940 \times 10^{-4}$
0·01	$4·972 \times 10^{-4}$	$2·88 \times 10^{-5}$	$6·350 \times 10^{-4}$	$4·910 \times 10^{-4}$
0·00625	$3·200 \times 10^{-4}$	$3·74 \times 10^{-7}$	$8·230 \times 10^{-6}$	$3·192 \times 10^{-4}$
0·005	$2·451 \times 10^{-4}$	$2·69 \times 10^{-6}$	$5·918 \times 10^{-9}$	$2·451 \times 10^{-4}$
0·00313	$1·753 \times 10^{-4}$	$5·14 \times 10^{-9}$	$1·131 \times 10^{-9}$	$1·753 \times 10^{-4}$
0·00157	$1·070 \times 10^{-4}$	$1·29 \times 10^{-9}$	$2·838 \times 10^{-10}$	$1·070 \times 10^{-4}$

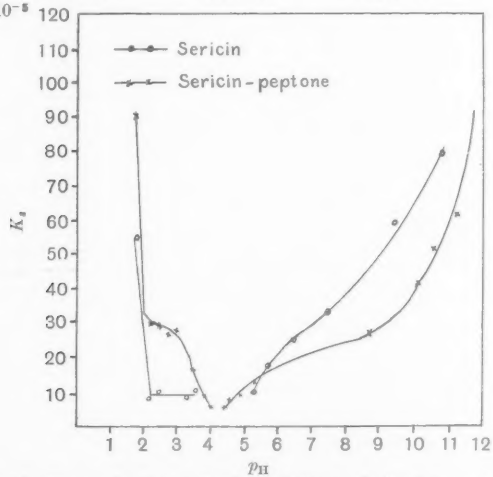

Fig. 6. The conductivity of sericin solution and of sericin-peptone solution at different p_H.

6. *The osmotic pressure of the sericin solution.*

The apparatus used was of Sørensen's type. The capacity of the collodion sack was 15 cc. The outside solution was 80 cc. of pure HCl or NaOH of such a concentration as to be half that used to make the sericin solution. The measurement was carried out in a thermostat at 25° and the reading was taken when the level of the liquid in the capillary no longer moved under a fixed opposing pressure. It took about 24–30 hours to attain equilibrium. At the same time the concentration of H ion in the outside and inside solutions was determined electrometrically. The pressure was expressed in millimetres of

water (see Fig. 7). The p_H values for the inside and outside solutions became equal at the point of minimum osmotic pressure, whilst the difference between these values changed sign on either side of this minimum. This held good both for sericin and sericin-peptone.

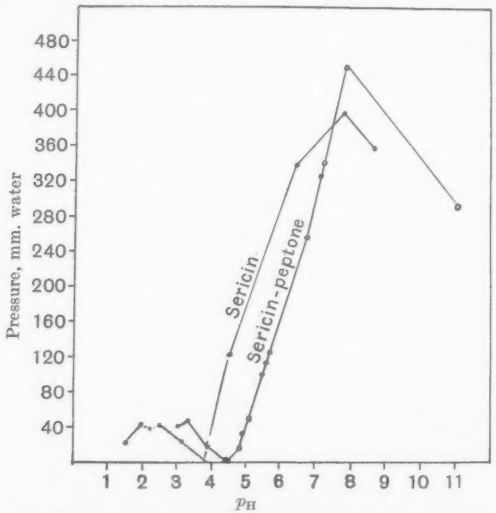

Fig. 7. The osmotic pressure of 1 % sericin and sericin-peptone solutions at different p_H.

7. *The change of some physico-chemical properties of sericin-HCl or -NaOH with time.*

In the investigation of the physico-chemical properties of proteins carried out by many investigators, little attention has been paid to the influence of time. Since the state of aggregation of the protein and the hydrolysis by acid or base are functions of time as well as of concentration, we must take the factor of time into consideration. This is especially the case when the solution of the protein is made from the solid state, as in the case of gelatin, caseinogen, sericin, etc. In the following experiments (Table IX) the change of the viscosity, the conductivity, the p_H and the precipitability at the isoelectric point with time were determined in solutions of 1 % sericin in 1/200 *M* HCl and 1/100 *M* NaOH (which were prepared as already described).

It is noticeable that the most remarkable change occurred in the viscosity. The acid sericin solution turned almost to a gel, while this was not the case with the sericin-NaOH solution.

III. The physico-chemical properties of sericin-peptone.

1. *The solubility of sericin-peptone.*

Sericin-peptone is readily soluble in hot water and is precipitated by 25 % alcohol or by half-saturation with ammonium sulphate. But in this process

of precipitation the H ion concentration plays a predominant rôle, as will be seen in the following experiment.

Table IX. *The change of some physico-chemical properties of sericin solutions with time at* 25°.

Hours	Viscosity	Conductivity	p_H	The amount not precipitated in 100 cc.
		(0·5 g. sericin + 50 cc. 1/200 HCl)		
0	1·41	$1·869 \times 10^{-2}$	1·45	74 mg.
½	1·42	$1·866 \times 10^{-2}$	1·43	74
1	1·43	$1·866 \times 10^{-2}$	1·42	80
2	1·45	$1·866 \times 10^{-2}$	1·39	79
4	1·56	$1·866 \times 10^{-2}$	1·38	78
8	1·83	$1·867 \times 10^{-2}$	1·38	82
16	2·96	$1·869 \times 10^{-2}$	1·36	84
32	4·12	$1·902 \times 10^{-2}$	1·32	86
		(0·5 g. sericin + 50 cc. 1/100 NaOH)		
0	2·63	$4·972 \times 10^{-4}$	9·46	0
½	2·67	$4·972 \times 10^{-4}$	9·46	0
2	2·68	$4·942 \times 10^{-4}$	9·45	0
4	2·70	$4·930 \times 10^{-4}$	9·43	0
8	2·74	$4·925 \times 10^{-4}$	9·43	0
16	2·80	$4·912 \times 10^{-4}$	9·42	8·6
32	2·86	$4·832 \times 10^{-4}$	9·40	10·9

A 1 % solution of sericin-peptone was prepared and dialysed against distilled water for 2 days, followed by electrodialysis[1]. The solution thus purified showed a specific conductivity of $0·756 \times 10^{-4}$ mho. During the electrodialysis some flocks appeared. They were filtered off so that the protein concentration was reduced to 0·953 %.

Into a series of test-tubes 1 cc. portions of the above sericin-peptone solution were pipetted out and diluted with 5 cc. portions of the acetate mixture of varying acidity (p_H 2·65–4·68) followed by the addition of 2 cc. portions of absolute alcohol. After being well stirred the turbidities were compared in the Kingslake nephelometer. The values in the following table were obtained by recalculating the nephelometrical readings on the basis that the precipitation of the protein at p_H 3·86 was complete (compare Table III).

Table X. *The precipitability of sericin-peptone by* 25 % *alcohol at different acidities.*

p_H	2·65	3·43	3·65	3·86	4·07	4·26	4·46	4·68	4·87
Sericin-peptone precipitated	0	0·824	0·886	0·953	0·913	0·834	0·620	0·552	0·0383

2. *The conductivity, p_H, viscosity and osmotic pressure of sericin-peptone-HCl and sericin-peptone-NaOH solution.*

The experiments and calculations were carried out (in the same way as described above) on the solutions prepared by mixing 25 cc. portions of sericin-peptone solution with 25 cc. portions of HCl or NaOH of various concentrations.

[1] The dialyser consisted of a U-tube made of hard glass. The limbs were connected by a ground joint and equipped with a collodion membrane at the bottom.

Table XI *a*. *The number of mols of HCl combined per* 0·2 *g. sericin-peptone in solutions of the given HCl concentrations containing* 1 *g. sericin-peptone per* 100 *cc.*

All the concentrations are given in mols per litre.

Total HCl conc. (a)	p_H	Free HCl from p_H (b)	$1/a$	b/a	HCl combined
0·05	1·39	$4·11 \times 10^{-2}$	1·035	$4·25 \times 10^{-2}$	$7·50 \times 10^{-3}$
0·025	1·75	$1·76 \times 10^{-2}$	1·025	$1·80 \times 10^{-2}$	$7·00 \times 10^{-3}$
0·0125	2·16	$6·90 \times 10^{-3}$	1·016	$7·01 \times 10^{-3}$	$5·49 \times 10^{-3}$
0·00625	2·64	$2·27 \times 10^{-3}$	1·010	$2·29 \times 10^{-3}$	$3·96 \times 10^{-3}$
0·008	2·43	$3·73 \times 10^{-3}$	1·013	$3·78 \times 10^{-3}$	$4·22 \times 10^{-3}$
0·0057	2·69	$2·06 \times 10^{-3}$	1·006	$2·07 \times 10^{-3}$	$3·63 \times 10^{-3}$
0·004	3·01	$9·68 \times 10^{-4}$	1·004	$9·72 \times 10^{-4}$	$3·03 \times 10^{-3}$
0·002	3·52	$3·02 \times 10^{-4}$	1·000	$3·02 \times 10^{-4}$	$1·70 \times 10^{-3}$
0·001	3·84	$1·44 \times 10^{-4}$	1·000	$1·44 \times 10^{-4}$	$0·865 \times 10^{-3}$
0·0005	4·04	$9·20 \times 10^{-5}$	1·000	$9·20 \times 10^{-5}$	$0·403 \times 10^{-3}$
0	4·36	$4·42 \times 10^{-5}$	1·000	$4·42 \times 10^{-5}$	—

Table XI *b*.

The number of mols of NaOH combined per 0·2 *g. sericin-peptone in solutions of the given NaOH concentrations containing* 1 *g. sericin-peptone per* 100 *cc.*

All the concentrations are given in mols per litre.

Total NaOH conc. (a)	p_H	p_{OH}	Free NaOH from p_{OH} (b)	$1/a$	b/a	NaOH combined
0·458	12·32	1·57	$2·69 \times 10^{-2}$	1·055	$2·84 \times 10^{-2}$	$1·74 \times 10^{-2}$
0·025	11·92	1·97	$1·07 \times 10^{-2}$	1·024	$1·09 \times 10^{-2}$	$1·41 \times 10^{-2}$
0·0125	11·38	2·51	$3·09 \times 10^{-3}$	1·000	$3·09 \times 10^{-3}$	$9·41 \times 10^{-3}$
0·00625	10·12	3·77	$1·70 \times 10^{-4}$	,,	$1·70 \times 10^{-4}$	$6·08 \times 10^{-3}$
0·008	10·58	3·31	$4·90 \times 10^{-4}$	,,	$4·90 \times 10^{-4}$	$7·51 \times 10^{-3}$
0·0057	9·96	3·93	$1·18 \times 10^{-4}$	,,	$1·18 \times 10^{-4}$	$5·58 \times 10^{-3}$
0·004	8·78	5·11	$7·76 \times 10^{-6}$	,,	$7·76 \times 10^{-6}$	$3·99 \times 10^{-3}$
0·002	5·22	8·47	$3·39 \times 10^{-9}$	,,	$3·39 \times 10^{-9}$	$2·00 \times 10^{-3}$
0·001	4·87	9·02	$9·55 \times 10^{-10}$	,,	$9·55 \times 10^{-10}$	$1·00 \times 10^{-3}$
0·0005	4·53	9·36	$4·37 \times 10^{-10}$	,,	$4·37 \times 10^{-10}$	$5·00 \times 10^{-4}$

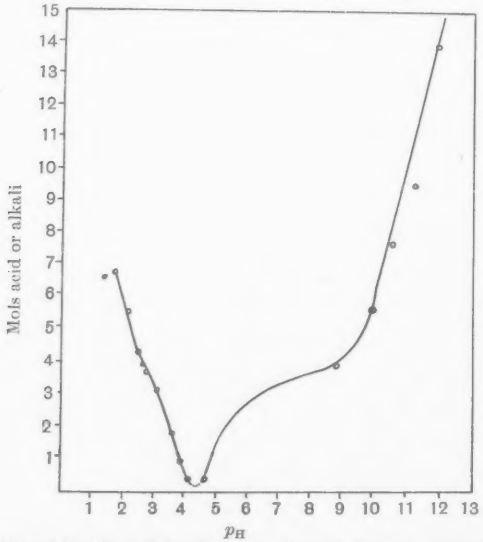

Fig. 8 The HCl and NaOH combination curve of sericin-peptone. Ordinates denote mols HCl or NaOH (multiplied by 10^3) which combine with 0·2 g. sericin-peptone.

Table XII. *The viscosities of sericin-peptone-HCl and -NaOH solutions.*

Sericin-peptone-HCl.									
p_H	1·39	1·75	2·16	2·64	2·43	3·01	3·52	3·84	4·04
Viscosity	1·03	1·03	1·04	1·03	1·03	1·05	1·05	1·04	1·03
Sericin-peptone-NaOH.									
p_H	4·36	4·53	4·87	5·22	8·78	9·96	10·58	11·38	12·32
Viscosity	1·04	1·03	1·04	1·04	1·04	1·05	1·05	1·04	1·04

The viscosity of the sericin-peptone solution showed no variation with change of acidity. These results seem to show that the sericin-peptone solutions are much more highly dispersed or more nearly molecular than the sericin solutions.

Table XIII *a* (Fig. 6).

The specific conductivities of sericin-peptone-HCl solutions.

Total HCl conc. mol	K_t	Free HCl conc. (*b*)	K_f	K_s due to sericin-peptone HCl
0·05	$2·168 \times 10^{-2}$	$4·11 \times 10^{-2}$	$1·734 \times 10^{-2}$	$4·340 \times 10^{-3}$
0·025	$8·333 \times 10^{-3}$	$1·76 \times 10^{-2}$	$7·427 \times 10^{-3}$	$9·060 \times 10^{-4}$
0·0125	$3·185 \times 10^{-3}$	$6·90 \times 10^{-3}$	$2·916 \times 10^{-4}$	$2·730 \times 10^{-4}$
0·00625	$1·195 \times 10^{-3}$	$2·27 \times 10^{-3}$	$9·579 \times 10^{-3}$	$2·381 \times 10^{-4}$
0·008	$1·841 \times 10^{-3}$	$3·73 \times 10^{-3}$	$1·574 \times 10^{-4}$	$2·670 \times 10^{-4}$
0·004	$6·674 \times 10^{-4}$	$9·68 \times 10^{-4}$	$4·085 \times 10^{-4}$	$2·589 \times 10^{-4}$
0·002	$2·867 \times 10^{-4}$	$3·02 \times 10^{-4}$	$1·275 \times 10^{-4}$	$1·592 \times 10^{-4}$
0·001	$1·515 \times 10^{-4}$	$1·44 \times 10^{-4}$	$6·080 \times 10^{-5}$	$9·070 \times 10^{-5}$
0·0005	$9·740 \times 10^{-5}$	$9·20 \times 10^{-5}$	$3·882 \times 10^{-5}$	$5·858 \times 10^{-5}$
0	$5·730 \times 10^{-5}$	—	—	$5·730 \times 10^{-5}$

Table XIII *b* (Fig. 6).

The specific conductivities of sericin-peptone-NaOH solutions.

Total NaOH conc. mol	K_t	Free NaOH conc. (*b*)	K_f	K_s due to sericin-peptone NaOH
0·04579	$8·700 \times 10^{-3}$	$2·69 \times 10^{-2}$	$5·919 \times 10^{-3}$	$2·781 \times 10^{-3}$
0·025	$4·161 \times 10^{-3}$	$1·07 \times 10^{-2}$	$2·354 \times 10^{-3}$	$1·807 \times 10^{-3}$
0·0125	$1·276 \times 10^{-3}$	$3·09 \times 10^{-3}$	$6·798 \times 10^{-4}$	$5·962 \times 10^{-4}$
0·00625	$4·183 \times 10^{-4}$	$1·70 \times 10^{-4}$	$3·736 \times 10^{-5}$	$4·023 \times 10^{-4}$
0·008	$6·101 \times 10^{-4}$	$4·90 \times 10^{-4}$	$1·077 \times 10^{-4}$	$5·024 \times 10^{-4}$
0·004	$2·500 \times 10^{-4}$	$7·76 \times 10^{-6}$	$1·708 \times 10^{-6}$	$2·482 \times 10^{-4}$
0·002	$1·173 \times 10^{-4}$	$3·39 \times 10^{-9}$	$7·454 \times 10^{-10}$	$1·173 \times 10^{-4}$
0·001	$8·620 \times 10^{-5}$	$9·55 \times 10^{-10}$	$2·100 \times 10^{-10}$	$8·620 \times 10^{-5}$
0·0005	$7·680 \times 10^{-5}$	$4·37 \times 10^{-10}$	$9·923 \times 10^{-11}$	$7·680 \times 10^{-5}$

Discussion.

The most characteristic property of sericin is that the isoelectric point lies further on the acid side than in the case of any other protein. The solubility-, viscosity-, and swelling-curves all point to a value near p_H 3·9. The experiments with the osmotic pressure, the combining capacity and the electrical conductivity also confirm the above statement, each being a minimum near that point.

As to the isoelectric point of sericin-peptone, there is a difference between the results obtained with the solubility in 25 % alcohol and with the osmotic pressure. In the former case the minimum occurs near p_H 3·9, in the latter

near p_H 4·4. Pauli [1922] observed also that the optimal precipitability of serum albumin and of gelatin by alcohol occurred at a little higher acidity than the minimum of the osmotic pressure. He explained this fact by assuming that the number of neutral particles of protein, which can be precipitated by alcohol, reaches a maximum at an acid concentration greater than the isoelectric point. But at the same time the osmotic pressure increases, because "the ionisation of $A^{n+}nCl^-$ (protein-chloride) gives rise to an increase in osmotic pressure which counteracts and finally exceeds the decrease caused by the formation of neutral particles." Besides this explanation it must be taken into consideration that the basic dissociation of an ampholyte like a protein is strongly suppressed in the presence of alcohol. Moreover, it has been confirmed that the greater the molecule of an ampholyte, the more it is possible to suppress the basic dissociation with diluted alcohol [Willstätter, 1922]. Therefore it may well be that the basic dissociation of sericin-peptone is diminished and the substance is more acidic even in so dilute an alcohol solution as 25 %. These considerations support the view derived from the experiment on the osmotic pressure that the true isoelectric point lies near p_H 4·4. It has been also asserted by many authors that the osmotic pressure of a protein solution can be mainly caused by the unequal distribution of electrolytes, which falls under the Donnan membrane equilibrium theory. If the protein molecule on one side of the membrane exists at its isoelectric point, there can be no unequal distribution of electrolytes. This was actually the case with sericin-peptone at p_H 4·4, where both the osmotic pressure and the unequal distribution vanished.

SUMMARY.

(1) The extraction of sericin in the autoclave is proved to deprive the protein molecule of its precipitability by dilute acid, accompanied by the disappearance of the glyoxylic acid reaction for tryptophan.

(2) A new method for the preparation of sericin is given.

(3) Several physico-chemical properties of sericin-HCl, sericin-NaOH, sericin-peptone-HCl and sericin-peptone-NaOH were examined, and it was found that the isoelectric points of sericin and sericin-peptone exist near p_H 3·9 and 4·4 respectively.

The author wishes to express his best thanks to Prof. Donnan for his kind advice and constant encouragement during the course of this work.

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CLV. THE COLORIMETRIC ESTIMATION OF MINUTE AMOUNTS OF COMPOUNDS OF SILICON, OF PHOSPHORUS AND OF ARSENIC.

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In the course of some work upon the minor constituents of sea-water and their utilisation by the phytoplankton it was necessary to make use of colorimetric methods and to study any possible interference of one constituent upon the method adopted for the analysis of another. Of the substances investigated the quantities present in sea-water are minute, being for phosphate, as P_2O_5, 1–40, for arsenic, as As_2O_3, about 25, and for silicate, as SiO_2, 40–200 mg. per m.^{3}, viz. per 1000 litres [Atkins, 1923, 1926; Orton, 1924].

The estimation of silicate in presence of arsenite, arsenate and phosphate.

The method of Diénert and Wandenbulcke [1923, 1924] may be used as follows: 2 cc. of 10 % ammonium molybdate and 4 drops of 50 % (by volume) sulphuric acid are added to 50 cc. of solution. A yellow colour results with solutions containing silicate. Permanent standards are obtained by dissolving 36·9 mg. of picric acid in a litre of water and diluting as required. The strength given corresponds to 50 mg. SiO_2 per litre. Thresh and Beale [1925] consider the method as accurate as the gravimetric, but use 40 mg. per litre of picric acid. Working with 100 cc. the writers used 8 drops of acid, but found that a blue tint was apt to develop. The trouble was traced to excess of acid. Taking only 4 drops, with sea-water, the solution is brought to p_H 2·1 and a clear yellow is obtained. The comparisons were made in Hehner tubes, using standards equivalent to 2·0, 0·5, 0·2 or 0·1 mg. SiO_2 per litre; the first is suitable for fresh waters, the others for sea-water. The limit detectable is close to 0·04 mg. per litre. For fresh or estuarine waters allowance has to be made for the tint already present which may be matched approximately against the picric acid standard.

Neither phosphates, arsenates nor arsenites, when present in moderate amount, give any colour with these reagents. Thus a solution of K_2HPO_4, 0·1916 g. per litre, gave no more tint than that due to 0·2 mg. per litre of SiO_2 and even this may have been derived from the glass of the bottle.

Similarly a solution containing 0·1612 g. per litre of As_2O_5 gave no perceptible tint. The method can accordingly be used for natural waters with no risk of error due to the substances named.

In solutions in which phosphates, arsenites or arsenates are suspected of vitiating the silicate results the latter may be estimated after evaporating down with hydrochloric acid and redissolving in sodium bicarbonate; alternatively the phosphate and arsenate may be removed by ferric chloride and ammonium hydroxide, and the arsenite similarly after oxidation with potassium chlorate and hydrochloric acid.

The estimation of phosphate, in presence of arsenite, arsenate and silicate.

Three colorimetric methods have been used: (*a*) Denigès [1920, 1921], using, in error, 50 % sulphuric acid by volume, instead of by weight; the modification was satisfactory, so was retained after the error had been pointed out. (*b*) Bell and Doisy [1920], also with Briggs' modifications [1922, 1924]. These need no description. (*c*) Pouget and Chouchak [1909, 1911]. All are methods which have been established for phosphate, but have not heretofore been used quantitatively for arsenic compounds, though Denigès, describing his qualitative test for phosphate, mentions a similar reaction—only about half as sensitive—with arsenate. Florentin [1921] also mentions possible interference of arsenate.

(*a*) *Denigès' method, as used.* To 100 cc. of phosphate solution add 2 cc. of reagent No. 1, followed by one drop of No. 2 and mix. No. 1 contains 100 cc. of 10 % ammonium molybdate with 300 cc. of 50 % (by volume) sulphuric acid. No. 2 is stannous chloride, freshly prepared from 0·1 g. tin, 1 drop 3 % copper sulphate solution and 2 cc. strong hydrochloric acid made up to 10 cc.

The blue colour which develops almost instantaneously is due to the reduction of a phosphomolybdic acid to a blue complex oxide of molybdenum [Vila, 1923; see also Hopkins, 1923]. Under the conditions of the test the excess of ammonium molybdate is not reduced, and so the intensity of the colour is proportional to the phosphate originally present and may be compared with a standard. The method is convenient and delicate, being suitable for sea-water, without concentration, and for soil extracts [Atkins, 1923, 1924, 1925]. Only the minimum amount of stannous chloride should be used. Toluene serves as a preservative for the standard phosphate solutions.

For comparison the Duboscq and Kober colorimeters were used, also Hehner tubes mounted on a nesslerising stand, with a sheet of cardboard in front of the tubes to screen them from extraneous light. On the opal plate of the stand a white card, with ruled lines, was arranged so as to form half of the field of each tube; the device is a considerable aid to accurate working. The triple field constituted by the Hehner tubes and the white plate, coupled with the greater length of the liquid columns, render work with these tubes

as accurate as with the more costly colorimeter and preferable for very dilute solutions.

Table I. *Estimation of arsenate, as* As_2O_5, *using Denigès' reagents and phosphate standards. All with Kober colorimeter, save first with Hehner tubes.*

Substance tested	Treatment	Taken mg. per 100 cc.	Found mg. per 100 cc.	Difference %
As_2O_5 (B.D.H.)	—	0·00806	0·0072	−11
,,	—	0·00806	0·0079	−2
As_4O_6 (B.D.H.)	Evaporated, with 10 cc. pure redistilled nitric acid, to dryness on sand-bath	0·00675	0·0058	−14
,,	As before but with "Arsenic test" nitric acid (B.D.H.)	0·00675	0·0068	+1
,,	Oxidation with 0·15 cc. of $N/10^3$ iodine solution, with bicarbonate	0·0067	0·0077	+14
,,	Oxidation with 0·15 cc. of $N/10^3$ iodine solution, with bicarbonate	0·0070	0·0065	−8

Table I shows the results obtained with solutions of arsenate, or of arsenite oxidised to arsenate. The amounts taken were very minute, so that the agreement between them and the quantities found is fairly satisfactory. It should be added that the arsenate solutions were compared with standard phosphate for convenience and because arsenic oxide is hygroscopic. The agreement between the amounts taken and found shows that this is legitimate, as might be expected from the fact that the "molybdenum blue" compound is the same whether derived from the reduction of phosphomolybdate or arsenomolybdate.

Arsenious oxide solutions were tested without oxidising, and though a blue colour was obtained, the results for As_4O_6 present were very low, showing only a partial reaction. The presence of arsenic pentoxide was suspected and Table II shows that the pure arsenite solutions do not give the Denigès reaction. The solutions, made up with bicarbonate, were all faintly alkaline and their reducing properties indicate that they have a tendency to spontaneous oxidation.

Though the foregoing conclusion appeared well established, it was thought that the presence of arsenate in the dilute arsenite solutions might be confirmed by titration with iodine before and after reduction with sulphurous acid. Such attempts were unsuccessful as even $N/10^5$ iodine is quite colourless.

Table II. *Estimation of arsenious acid, as* As_4O_6, *using Denigès' reagents and phosphate standard. All with Kober colorimeter, save first with Hehner tubes.*

Substance tested	Taken mg.	Found mg.	Difference %
As_4O_6 Kahlbaum, 99·98 % (for analysis)	0·0049	0·0011	−77
,,	0·0195	0·0057	−71
,,	0·0195	0·0056	−71
As_4O_6 Kahlbaum resublimed	0·0070	0·0005	−93
As_4O_6 Kahlbaum resublimed, solution 14 days later	0·0070	0·0010	−86
As_4O_6 Kahlbaum resublimed	0·0700	0·0044	−94
,, ,,	0·0700	0·0045	−94
As_4O_6 (B.D.H.)	0·0675	0·0015	−98

In Table I results obtained by oxidising arsenious acid with nitric acid have been shown, but this requires care. It was accomplished by evaporating the solution to dryness in a beaker on a sand-bath. It is necessary to remove the beaker immediately dryness has been reached, otherwise low results are obtained. Care must be taken to avoid "sputtering," but the use of a conical flask is inadmissible, as to remove all acid too high a temperature has to be used. In oxidising with iodine, adding bicarbonate, a large excess of the reagents must be avoided. Blank determinations were made upon all the reagents used for the purposes of this research.

Oxidation by means of hydrogen peroxide was also attempted, the latter being then decomposed by boiling, but on adding the Denigès reagents a greenish-yellow developed, due to the ammonium molybdate alone; the colour can be destroyed by stannous chloride, but a turbidity remains. Blanks carried out with hydrogen peroxide showed that it was necessary to heat to dryness and then quite strongly before a residue could be obtained which gave no colour with ammonium molybdate solution. The hydrogen peroxide, stated to be free from barium salts, probably contained traces of barium peroxide; phosphate, also said to be absent, was present to about one part per million. The heating of the residue is not desirable in the presence of arsenic compounds. Electrolytically prepared peroxide gave no better results.

Silicate up to 8 mg. SiO_2 per litre gives no reaction with the Denigès reagents and so does not lead to an over-estimate for phosphate. Higher concentrations were not examined.

(*b*) *Bell and Doisy's method* for determining phosphate was tested for the estimation of arsenate. A trial was first made with two phosphate solutions of known strength, assuming one to be the standard. Taking 0·500 mg. in 50 cc., 0·517 mg. was found; difference + 3·4 %. Taking 0·0201 mg. of arsenic oxide, the amount found was 0·0208; difference + 3·5 %. The reaction is therefore applicable to the estimation of arsenates also.

Using solutions of arsenious oxide varying shades of green and blue were obtained. These it was impossible to match against the phosphate standards. It should be possible to oxidise to arsenate and then to estimate, but this was not tried.

(*c*) *Pouget and Chouchak's method.* The phosphate solution is acidified with nitric acid and to it is added sodium molybdate similarly acidified; these are followed by a filtered neutral solution of strychnine sulphate. After energetic shaking the yellow opalescence which appears is compared with a similarly treated standard solution. The method is less sensitive than is that of Denigès. Matthews [1916, 1917] found the reaction suitable for work with sea-water, but the process is lengthy as the phosphate has to be precipitated with ferric chloride from 500 cc.

Pouget and Chouchak state that arsenic acid gives no precipitate with strychnine molybdate under the conditions of the determination, but its presence is disturbing when the weight of arsenic attains two or three times

that of phosphorus. Their method does not involve warming the solution. Matthews carried out the estimation in presence of arsenious acid, without oxidising, and found that it did not interfere with the accuracy of the analysis.

Certain experiments carried out by Matthews after oxidation suggested to us the presence of arsenious compounds, rather than phosphorus in organic combination. Accordingly qualitative tests were performed upon solutions of arsenite, arsenate and phosphate, blanks being carried out simultaneously upon the reagents.

The results of these and other qualitative tests are shown in paragraphs 6–8 of the Summary.

Summary.

1. The reagents used by Diénert and Wandenbulcke for estimating silicate give no colour with any moderate quantities of phosphate, arsenate or arsenite.

2. The amounts of phosphate and arsenate ordinarily present in natural waters do not suffice to interfere with the method of Diénert and Wandenbulcke for silicate.

3. The method of Denigès for phosphate may be employed:

(*a*) for estimation down to 0·0002–0·0004 mg. of P_2O_5 in 100 cc.;

(*b*) for estimation of equivalent quantities of arsenate, as As_2O_5, but with arsenite it gives a faint colour only, which may be attributed to the presence of arsenate.

4. The amounts of silicate ordinarily found in natural waters do not interfere with the accuracy of Denigès' method for estimating the phosphate.

5. Bell and Doisy's method does not appear to be as convenient or as delicate as that of Denigès for the estimation of phosphate. It can be used for arsenate also, but solutions of arsenious acid give very irregular results.

6. The reaction of Pouget and Chouchak for phosphates is not given by arsenites, save very faintly on warming, due apparently to arsenate present. It is only given very faintly in the cold with arsenates, but on heating turbidity and a yellow colour develop. With phosphate a strong colour and a marked turbidity appear in the cold immediately, save with very dilute solutions, and become more intense on heating.

7. The Denigès reaction is not given by glycerophosphate, save in so far as free phosphate is present; triphenyl phosphate does not give the reaction, nor is it readily hydrolysed on boiling the aqueous solution as is glycerophosphate; tricresyl phosphate does not give the reaction even after prolonged boiling with water.

8. The Denigès reaction is not given by vanadates, by tungstates or by phosphotungstates. With the latter however a purple tint is slowly produced, but this is not a sensitive reaction.

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CLVI. THE PHYSIOLOGICAL RÔLE OF VITAMIN B. PART I.

THE RELATION OF VITAMIN B TO TISSUE OXIDATIONS.

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Introduction.

In spite of a considerable amount of investigation and the accumulation of an extensive literature, the problem of the part played in the economy of the organism by the dietary principle known as vitamin B remains unsolved. Nevertheless, there has been no dearth of expressed opinions as to the rôle of this factor or regarding the cause of the nutritive failure which follows its withdrawal from the dietary. Indeed, the very multiplicity of theories and the wide field over which they range seems strong evidence that the actual facts have not been disclosed.

Reviewing the many hypotheses that have been advanced, and searching for some outstanding feature, one is impressed by the frequency with which investigators have expressed a belief that vitamin B is concerned in maintaining the normal level of activity of the oxidising mechanisms of the body, although there exists considerable diversity of opinion regarding the actual cause of the lowered oxidation.

In one direction there is a fairly general agreement that the animal deprived of vitamin B shows a markedly reduced oxygen utilisation [Raomino, 1916; Abderhalden, 1920, 1921, 1, 2; Anderson and Kulp, 1922; Caridroit, 1922; Mattill, 1923, 1, 2; Gulick, 1922, 1924; Japanese references cited by Shinoda, 1924, 1, 2]. Apart from a more or less general agreement on this point, there are not only divergent opinions regarding the significance of certain changes in the R.Q. that have been noted during the course of the condition induced by the deficiency, but also various suggestions as to the cause of the alleged lowered consumption of oxygen. The majority of investigators seem to have been inclined to attribute it, wholly or in part, to an actual reduction in the amount or the activity of the oxidising enzymes.

Amongst such suggestions is that advanced by Dutcher [1918], based on his estimations of catalase in normal and "beri-beri" tissues, that the vitamin stimulates the production of this enzyme. A somewhat similar view is taken by

Findlay [1921, 1] concerning a relationship between vitamin B and glyoxalase. The broader view is held by Hess [1921, 1922] who, insisting on the essential similarity between "beri-beri" induced by a diet of polished rice in pigeons and the condition following the administration of sub-lethal doses of cyanides, regards the nutritive failure in the former state as being due to a reduction in the amount or efficiency of the oxidising enzymes. From these views we may, bearing in mind Warburg's theories regarding the rôle of iron in biological oxidations, pass naturally to such suggestions as that one form of beri-beri in animals may be due to a deficiency of iron [McGowan and Crichton, 1924]. Numerous other theories might be quoted, many of which, such as the view that the animal suffering from a deprivation of vitamin B cannot utilise carbohydrates (see later references), could be loosely woven in with the theories of decreased tissue oxidation until an attractive hypothesis regarding the rôle of vitamin B had been built up.

However, the literature contained so many contradictions and discrepancies and a critical analysis revealed that so much of the experimental work recorded seemed unsatisfactory, that we were led to commence the investigation on which we are now reporting. Perhaps it should be remarked at this point that at the outset of the investigation we accepted as the foundation of our plans the one statement upon which there seemed to be a fair amount of agreement, namely, that deprivation of vitamin B leads to a reduction of the oxygen utilisation of the whole animal. In view of this fact, it seems the wiser plan to give an account of our experiments in the order in which they were carried out, rather than in what would seem the more logical sequence.

Anaerobic reduction of methylene blue by normal and "beri-beri" tissues.

The success which has followed the extensive use of methylene blue as a hydrogen acceptor to serve as an indicator in studies of anaerobic oxidation-reduction processes prompted us to compare the rates of reduction of this dyestuff by normal tissues and those from "beri-beri" animals under the conditions outlined by Thunberg [1920].

By employing the somewhat similar technique described by Lipschitz [1921], in which *m*-dinitrobenzene is used as the indicator, Hess [1921, 1922, 1923] observed that the rate of reduction was markedly reduced in tissues from "beri-beri" pigeons.

These results were confirmed by Hess and Messerle [1922] and by the work of Rohr [1923], who traced a parallel between the power of tissues to reduce this dyestuff and their antineuritic potency.

In our experiments a large number of observations were made on the time of reduction of methylene blue by the tissues before and after washing free from water-soluble extractable substances, and by washed tissue preparations in the presence of an artificial "metabolite" (succinate), the details of the Thunberg technique being closely followed in all respects.

The following results are typical of those obtained with tissues removed immediately after killing from a normal pigeon and one exhibiting typical symptoms of avian polyneuritis.

Table I. *Times of decoloration of methylene blue by pigeon breast muscle.*

Buffer p_H 7·5 (phosphate). Methylene blue 1 in 5000. Temp. 35°.
Weight of tissue 0·2 g. K succinate $M/10$.

	Buffer cc.	K succinate $M/10$ cc.	Methylene blue cc.	Time
Unwashed tissue				
Normal	2·0	—	1·0	12·0
	2·0	—	1·0	11·5 } 12·2 mins.
	2·0	—	1·0	13·0
"Beri-beri"	2·0	—	1·0	16·0
	2·0	—	1·0	14·5 } 14·3 mins.
	2·0	—	1·0	12·5
Washed tissue				
Normal	1·4	—	0·2	
	1·4	—	0·2	Over 2 hours
	1·4	—	0·2	
	1·0	0·4	0·2	12·0
	1·0	0·4	0·2	11·5 } 11·5 mins.
	1·0	0·4	0·2	11·0
"Beri-beri"	1·4	—	0·2	
	1·4	—	0·2	Over 2 hours
	1·4	—	0·2	
	1·0	0·4	0·2	12·0
	1·0	0·4	0·2	11·5 } 11·75 mins.

These results are confirmed by those in Table II, which are typical of those obtained in a number of studies on the leg muscles of normal rats and others in the final stages of the nutritive disorder induced by a diet deficient in vitamin B.

Table II. *Times of decoloration of methylene blue by rat leg muscle.*

Buffer p_H 7·5 (phosphate). Methylene blue 1 in 5000. Temp. 35°.
Weight of tissue 0·2 g. K succinate $M/10$.

	Buffer cc.	K succinate $M/10$ cc.	Methylene blue cc.	Time
Unwashed tissue				
Normal	2·0	—	1·0	17·5
	2.0	—	1.0	14.5 } 16·3 mins.
	2·0	—	1·0	17·0
"Beri-beri"	2·0	—	1·0	15·5
	2·0	—	1·0	16·5 } 16·5 mins.
	2·0	—	1·0	17·5
Washed tissue				
Normal	1·4	—	0·2	86·0
	1·4	—	0·2	72·0 } 80·0 mins.
	1·4	—	0·2	81·0
	1·0	0·4	0·2	19·0
	1·0	0·4	0·2	20·0 } 19·0 mins.
	1·0	0·4	0·2	18·5
"Beri-beri"	1·4	—	0·2	95·5 } 93·0 mins.
	1·4	—	0·2	91·0
	1·0	0·4	0·2	15·0
	1·0	0·4	0·2	14·5 } 16·0 mins.
	1·0	0·4	0·2	18·5

In our opinion these figures conclusively demonstrate that the system responsible for the decoloration of the dyestuff is operating with a normal efficiency in the muscle tissues of pigeons and rats suffering from vitamin B-deficiency.

In spite of the apparent normality of the "beri-beri" tissues as regards their power to reduce methylene blue, it seemed of interest to examine whether the addition of small quantities of yeast extract accelerated the change, particularly in view of the emphatic claim of Abderhalden and his colleagues, to which detailed reference will be made later, that such an addition promptly and markedly increases the respiration of isolated tissue preparations, especially those from "beri-beri" animals.

As will be seen from the figures in Table III, we could trace no stimulating effect on adding yeast extract to the Thunberg tests; the slight acceleration of reduction when the yeast extract was added to the washed tissue preparation alone was, of course, to have been expected, and merely indicated the activation of certain constituents of the extract capable of yielding hydrogen to the dyestuff.

Table III. *Times of decoloration of methylene blue by pigeon breast muscle.*

Effect of yeast extract on rate of decoloration of methylene blue by K succinate and washed muscle.
Buffer p_H 7·5 (phosphate). Methylene blue 1 in 5000. Temp. 35°.
Weight of tissue 0·2 g. K succinate $M/10$.

Washed tissue	Buffer cc.	Methylene blue cc.	K succinate cc.	2·5 % yeast extract cc.	Time
	1·4	0·2	—	—	About 3 hours
	1·4	0·2	—	—	
	1·4	0·2	—	—	
	1·4	0·2	—	0·2	32 } 31 mins.
	1·4	0·2	—	0·2	30 }
	1·0	0·2	0·4	—	10 }
	1·0	0·2	0·4	—	11 } 10·3 mins.
	1·0	0·2	0·4	—	10 }
	1·0	0·2	0·4	0·2	12 }
	1·0	0·2	0·4	0·2	10 } 11 mins.
	1·0	0·2	0·4	0·2	11 }

Convinced from these experiments that the oxidative activity of the muscle tissue, in so far as it can be revealed by a technique of this type, is normal in animals exhibiting the typical symptoms associated with the later stages of vitamin B-deficiency, it remained to be seen whether the same would be found to be true in the case of animals poisoned with cyanide. Hess [1921] has described how by the intra-muscular injection of sub-lethal doses of cyanides it is possible to produce a condition in pigeons which he alleges closely resembles that caused by prolonged feeding on polished rice. His views have been attacked by Abderhalden [1922] who refuses to admit that there is any real relation between the two conditions on the grounds that cyanides do not depress, but may actually raise, the gaseous metabolism during the period when the symptoms are apparent, and also because yeast extracts will not prevent or cure the condition brought about by cyanides.

A number of experiments have been made by us along these lines, and on some points we can confirm the observations recorded by Hess. The administration of doses of KCN of about 0·4 mg. per 100 g. of body-weight is followed after some 4 minutes by very rapid collapse and death. Smaller doses, 0·1–0·25 mg. per 100 g. weight, give rise to staggering some 4 or 5 minutes after injection, after which the bird sits still with eyes shut, ruffled feathers, and gives occasional gasps. Some 10 minutes later there is usually recovery. With intermediate doses, that is, 0·25–0·35 mg. per 100 g. weight, these symptoms are exaggerated and followed by convulsions resembling in some respects the spasms shown by "beri-beri" pigeons. From these the birds sometimes recover.

In Table IV are given the data obtained from a study of the breast muscle of a pigeon which had received three injections of a freshly prepared solution of potassium cyanide. The bird weighed 370 g. and received a dose of 0·35 cc. of freshly prepared 0·1 % solution of KCN at 10.52 a.m. At 11.25 a.m., having recovered, it received a dose of 0·6 cc. and at 11.37 a.m. a dose of 1·0 cc. This last dose produced severe convulsions during which the animal was killed and the breast muscle from the opposite side to that into which the injections had been made dissected out for immediate use.

Table IV. *Time of decoloration of methylene blue by "cyanide" pigeon muscle.*

Three sub-lethal doses of KCN in the left breast (totalling 1·95 mg.). Weight of bird 370 g. Muscle cut from the *right* breast. Weight of tissue 0·2 g.

	Buffer cc.	Methylene blue cc.	K succinate cc.	Time	
Unwashed tissue	2·0	1·0	—	12·0	
	2·0	1·0	—	10·0	10·5 mins.
	2·0	1·0	—	9·5	
		(Normal unwashed—12·2 mins.)			
Washed tissue	1·4	0·2	—		
	1·4	0·2	—	Over 2 hours	
	1·4	0·2	—		
	1·0	0·2	0·4	14·5	
	1·0	0·2	0·4	13·5	14·1 mins.
	1·0	0·2	0·4	14·5	
		(Normal washed—11·5) mins.			

It is doubtful whether the rate of reduction by washed tissue in the presence of succinate is actually slower than in the case of normal muscle as the figures are, in the light of our experience, scarcely outside the range of variation that may be expected in duplicate experiments.

It is not intended to discuss at this point the possible relations between the cyanide poisoning and "beri-beri," but merely to remark that, having satisfied ourselves that muscle tissue removed from animals almost moribund in both conditions shows normal oxidative activity, in so far as this is revealed by the power to reduce methylene blue under anaerobic conditions in the presence of normal metabolites or added succinate, it seemed desirable to examine the actual oxygen consumption of the tissues by a direct method.

Oxygen consumption of normal and "beri-beri" tissues.

In this connection the literature provided two opposing claims. Abderhalden and his colleagues in a series of papers record a large number of experiments to show that in the absence of extracts supposed to contain vitamin B the respiration of both plant and animal cells is greatly reduced. Abderhalden [1920, 1921, 1, 2; Abderhalden and Wertheimer, 1921, 1, 2, 1922] observed a reduced oxygen uptake of pigeons that had been fed on polished rice, and has many times since emphasised not only this point, but also his conviction that the oxygen utilisation rapidly rises after the administration of yeast preparations. Furthermore, Abderhalden and Schmidt [1920] presented data to show that whereas the respiration of tissues removed from pigeons fed on polished rice was much below normal, it could be raised by the addition *in vitro* of extracts believed to contain vitamin B. Somewhat similar but rather unconvincing results are reported by Shinoda [1924, 2].

In complete disagreement with this claim are the results of experiments by Roche [1925] who found that isolated tissues of both "beri-beri" and starving pigeons showed an oxygen uptake of the same order as that of tissues removed from the normally fed control animals. As will be seen, our observations entirely support Roche.

For studying the oxygen uptake of isolated tissue preparations we employed a modified Barcroft-Haldane apparatus of which the respiration vessels were specially constructed with flattened bottoms so as to expose a relatively large surface of a very shallow layer of tissue suspension to the air. The potash for absorbing carbon dioxide was contained in a bulb so blown on the side of the neck of the vessel as to give a relatively large absorbing surface without risk of splashing during shaking. From a number of experiments we have selected the typical curves shown in Figs. 1, 2, 3 and 4.

Fig. 1 illustrates the identical rate of absorption of oxygen by normal and "beri-beri" breast muscle of the pigeon under the conditions of the experiment; that is, suspended in phosphate buffer of p_H 7·4, and shaken vigorously in a bath at 37°. Much the same types of curves are shown in Fig. 4, where is also given the curve illustrating the rate of oxygen uptake of the breast muscle of a pigeon poisoned with potassium cyanide; actually in this case the bird described on p. 1233. Moreover, this chart illustrates how the addition of yeast extract failed in our experiments to make any alteration of the slope of the curve of the "beri-beri" muscle. Fig. 3 represents the rates of oxygen uptake of liver tissue from normal and "beri-beri" pigeons, which, if allowance is made for a slight lag of the "beri-beri" liver in the early stages of the experiment, are quite similar.

The same is also true of the muscle from acutely and chronically poisoned "cyanide" pigeons in Fig. 2, for in view of other results we have obtained, such as those in Fig. 4, we are not inclined to attach any importance to the slight divergencies of these curves. At any rate, considering that the tissues compared with the normal preparations were removed from animals showing

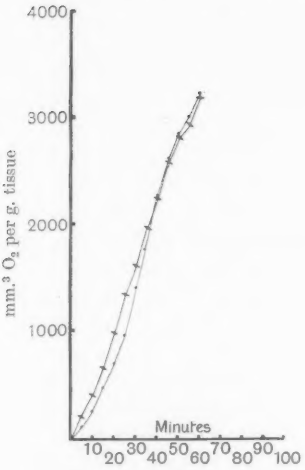

Fig. 1.

Breast muscle.
p_H 7·5. T. 35°.
"Beri-beri" |—|—|
Normal •—•—•

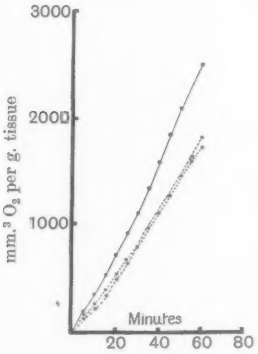

Fig. 2.

Breast muscle.
•—•—• Normal.
•– – –•– – –• Chronic cyanide poisoning.
•. . . .•. . . .• Acute cyanide poisoning.

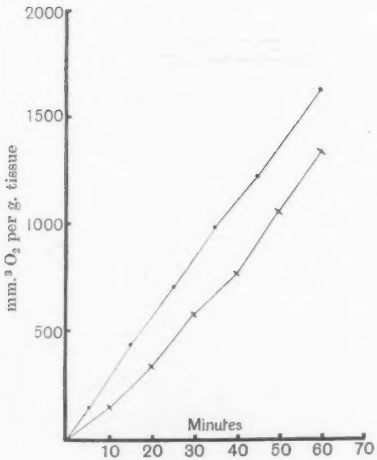

Fig. 3.

Liver tissue
p_H 7·5
Temp. 35°.
"Beri-Beri" |—|—|
Normal •—•—•

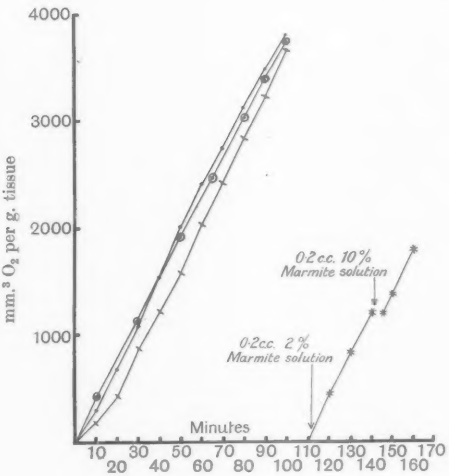

Fig. 4.

Breast muscle
•—•—• Normal.
|—|—|—| "Beri-beri."
⊙—⊙—⊙ Cyanide.
——* "Beri-beri" and marmite.

either severe symptoms of "beri-beri" or practically dying from cyanide poisoning, any reduction of oxidative activity which they show seems to us to be relatively insignificant, and out of all proportion to what one would expect from the striking figures presented by Abderhalden and his colleagues[1].

As will be appreciated from Figs. 1–4, our experiments led us definitely to reject the results of Abderhalden, and to conclude that the tissues themselves show no appreciable impairment of oxidative activity in vitamin B-deficiency.

How this conclusion stands with reference to much of the previous work on the subject will be left over until the discussion. Meanwhile, tracing the course our experiments followed, we shall describe how our failure to confirm Abderhalden led us to investigate the oxygen requirements of the intact animal in vitamin B-deficiency, for it seemed at this stage of our enquiry an entirely reasonable hypothesis that, whereas the tissues themselves might be in possession of a normal equipment of oxidative agents, there might well be in the whole organism, in the absence of vitamin B, some incoordination of the mechanism for controlling metabolism that would result in lowered gas exchange. To gain light on this point we made a prolonged study of the gaseous metabolism of rats in various states of nutrition.

Influence of state of nutrition on oxygen uptake of rats.

At an early stage we were obliged to recognise that, whereas it may be an easy matter to determine the respiratory quotients of a small animal, it is far from simple to obtain values for its oxygen uptake which are significant. The obvious source of trouble, and curiously it is one which many investigators in this field seem either to have neglected entirely or to have appreciated inadequately, is the difficulty of controlling the movements of the animal in the chamber. Preliminary studies with both normal and "beri-beri" animals gave such scattered values that, apart from a clear indication that the gas exchange was greatly depressed in the last stage of the vitamin B-deficiency, nothing of certain value could be deduced from them.

For estimating the oxygen utilisation of the rats we employed a closed circuit system in which was placed a recording spirometer. The general plan of the apparatus is shown in Fig. 5. The apparatus was, as far as possible, immersed in a bath at constant temperature. The animal having been placed in the chamber *A*, which also contains absorbents in the form of calcium chloride and soda lime beneath the platform, the air was caused to circulate by means of the pump *B*, consisting of a rubber bulb with inlet and outlet valves and compressed at the desired frequency by the eccentrically driven plunger *C*. By means of a three-way tap a known volume of oxygen could be passed by displacement with mercury into the circuit from the container *D*, and estimations of the rate of utilisation of the gas were made by noting the time taken for the writing lever of the spirometer, which rose on admission of the gas, to fall once again to the base line on the recording drum *E*.

[1] Estimations of moisture show that the results obtained by us cannot be attributed to changes in the water content of the tissues.

In spite of the fact that all rats were starved for at least 24 hours before making a record of their oxygen consumption, our earlier results showed so wide a variation that it seemed necessary to have some sort of base line to which our figures could be referred. This we obtained by a series of estimations of the oxygen consumption of rats starved for 24 hours and placed under amytal (ethyl-*iso*-amylbarbituric acid) anaesthesia. It is, of course, necessary to maintain the body temperature of the animal during amytal anaesthesia if normal values for the oxygen consumption are to be obtained, and in all experiments of this type, unless otherwise desired, the temperature of the bath was kept at 38°. The figures obtained on a number of rats of different weights are plotted in Fig. 6, and through them a reasonable curve may be traced. Our results confirm the opinion expressed by Deuel and Chambers [1925] that gaseous exchange experiments on animals under the influence of amytal provide trustworthy information concerning the normal body metabolism.

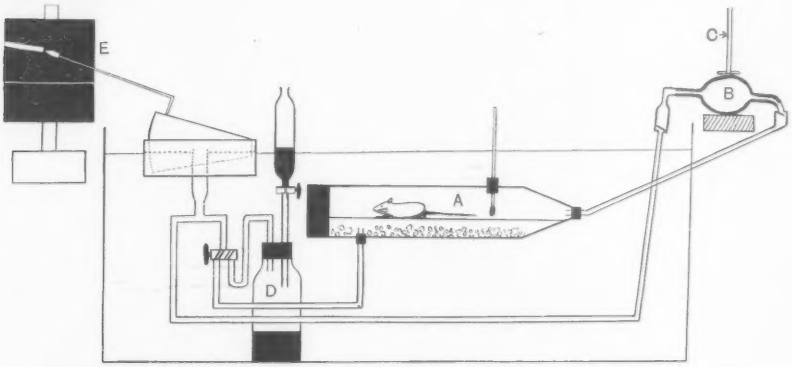

Fig. 5.

It is, of course, important to administer approximately the same dose of the anaesthetic to each animal. We usually employed 10 mg. per 100 g. of body-weight injected subcutaneously. This dose produced a light anaesthesia and enabled us to obtain good reproducible readings of oxygen consumption. The figures thus obtained are naturally on a slightly lower level than that of the values for what is usually regarded as the basal metabolism.

Accepting the results of the experiments with amytal, we were able to construct another curve, the upper one in Fig. 6, which was traced parallel to the "amytal" curve and traversing the area occupied by points representing the oxygen uptake of non-anaesthetised animals in all experiments where little or no movement of the rats in the chamber was observed. This seemed to us, in view of the very great difficulties with which we were faced, a justifiable means of obtaining some sort of estimate of the basal metabolism of rats of different weights; at any rate, it provided a method of which the error was probably smaller than the differences we expected to detect.

Later in this investigation the question of the effect of the environmental temperature on the intensity of the metabolism assumed some importance, and it was decided to compare the oxygen uptake of rats at 38° and 28°: the normal body temperature of the rat being about 38°. Generally speaking, we found in studying normal rats that the errors due to movement of the animals were greater than the difference between the metabolism at the two temperatures, so that the attempt to construct separate curves was abandoned.

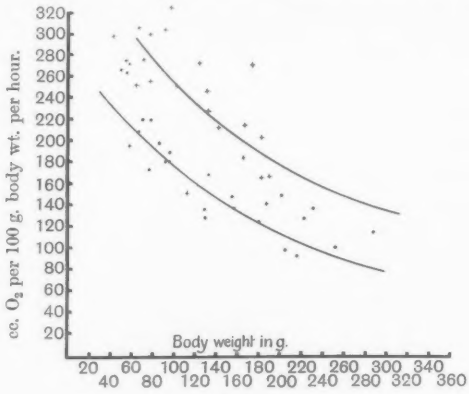

Fig. 6.

Oxygen consumption of normal rats.
Temp. 38°
• Under amytal.
+ Unanaesthetised.

Oxygen consumption of rats deprived of vitamin B.

Groups of young rats were fed on a vitamin B-deficient diet employed in this laboratory, and composed as follows:

Extracted caseinogen	20 parts
,, rice starch	63 ,,
Salt mixture	5 ,,
Cod-liver oil	2 ,,
Hardened vegetable oil	10 ,,

After various periods on this diet they were starved for 24–36 hours and determinations of their oxygen consumption made. The results were widely varying values, from an analysis of which in the light of the curves in Fig. 6 it seemed clear that the gaseous exchange was not reduced appreciably in vitamin B-deficiency, except during a comparatively short period before death.

It seems necessary before presenting a selection of these results to draw attention to certain features of the clinical picture exhibited by rats deprived of vitamin B. The deprivation, as is well known, results in an almost immediate cessation of growth in the young animal, followed by a period during which its

weight either is maintained or slowly falls. The duration of this period is roughly proportional to the initial weight of the animal, and during this time the rat seldom shows obvious symptoms of ill health, other than loss of appetite, constipation, disinclination to exercise, ruffled fur, and a slight fall in pulse rate and body temperature. Quite suddenly the last phase of the disorder appears, body temperature shows a rapid fall, respiration becomes laboured, there may be incoordination of the hind limbs followed by convulsions and death; usually this phase lasts some 24 hours, seldom more than 48.

It is particularly the body temperature of these animals to which we wish to draw attention, because the fall that has been so often observed has been regarded by some authorities as characteristic of vitamin B-deficiency. To this question we will return again later, but its immediate interest is in connection with our observation that only during the last stage of the deprivation, when the body temperature of the animals was falling rapidly, were low values obtained for oxygen consumption markedly outside the range of variation of normal figures.

This is seen from the figures given in Table V, in which are recorded oxygen consumptions of rats fed on vitamin B-deficient diet for different periods and measured at an environmental temperature of 28° after 24 hours' starvation.

Table V. *Oxygen consumption of rats on vitamin B-deficient diet.*

Weight g.	Days on diet	Rectal temperature ° C.	Oxygen uptake cc. per 100 g. per hour	Approximate normal value (±20) cc. per 100 g. per hour
49	35	30·5	120	285
50	21	35·0	166	285
50	—	31·0	222	285
54	35	31·5	185	280
56	25	31·0	133	280
65	7	35·8	293	278
75	35	33·9	200	260
77	35	33·9	260	260
80	21	35·8	251	260
132	35	32·2	151	220
147	35	34·0	291	210
170	21	36·1	166	190
174	21	36·0	156	190
201	14	35·5	143	180
203	6	36·6	163	117

Generally speaking, it was observed that definitely low oxygen consumptions were recorded when the body temperature of the rats had fallen below 33°, and it remained for the significance of this fact to be considered.

The fall of body temperature in a warm-blooded animal might arise from one or more of several causes. On the one hand, it might be due to a breakdown of some part of the nervous or glandular apparatus for temperature regulation, whilst, on the other hand, it might follow a marked reduction in the intensity of the oxidations in the tissues. We have seen that the results of the experiments on the oxygen uptake of tissue suspensions are not in favour of the view

that the mechanisms for carrying out cellular oxidations are impaired in vitamin B-deficiency.

An attempt to throw additional light on this question was made by investigating the influence on metabolism of raising the environmental temperature of animals showing a reduced body temperature and oxygen uptake in the last stages of the condition induced by deficiency of vitamin B. In most cases after warming the animal up to its normal body temperature by placing it in a warm chamber for 2 or more hours, the oxygen consumption rose to a figure approaching the normal level; coincidently there was very marked but only temporary, improvement in the general condition of the animal.

Table VI.

Weight g.	Rectal temperature at beginning of experiment ° C.	Temperature after warming up ° C.	Oxygen uptake in cc. per 100 g. weight per hour		
			At 28°	At 38°	Normal ±20
49	30·5	37·5	120	235	285
54	31·5	37·3	185	262	280
56	31·0	37·3	133	233	280

These results seem definitely to confirm those of the experiments on the oxygen utilisation of the isolated tissues in showing that mechanisms for cellular oxidations are unimpaired by vitamin B-deficiency. They are also obviously related to the interesting facts recorded by Peters [1924, 1925] that the well-known symptoms of vitamin B-deficiency in pigeons occur earlier in birds exposed to cold than in those kept in a warm room, and also that temporary cures of pigeons showing signs of imminent convulsions may be effected by bringing them into warmer surroundings. It occurred to us that by maintaining rats fed on vitamin B-deficient diets in an environment of a temperature approximating to their normal body temperature the final decline might be postponed. A few experiments, admittedly inadequate, seemed to indicate that such was not the case.

While considering how best to investigate the cause of the fall of body temperature in the vitamin B-deficient animals, we were more and more impressed with the necessity for controlling the influence on the organism of the greatly diminished food consumption which is so characteristic a consequence of deprivation of vitamin B, but before describing the results obtained in that part of the investigation, we may briefly refer in passing to a few tests which were made in order to determine whether the atrophy of the thyroid which numerous authorities have noted in vitamin B-starved animals [Funk and Douglas, 1914; McCarrison, 1919, 1, 2; Findlay, 1921, 2; and others] might in any manner be connected with the fall of body temperature. Dutcher [1920] thought that some relief of symptoms in pigeons followed administration of thyroid gland or thyroxine. The tests on thyroid administration were not carried very far, as the investigation passed to the more important path we have just mentioned, but confirmation was obtained of the observations pre-

viously reported by Drummond [1918], that oral administration of desiccated thyroid gland in daily doses of about 100 mg. tended, if anything, to hasten the appearance of the fatal decline. No improvement of symptoms or rise of body temperature was noted in the animals treated with the gland preparation.

Relation of vitamin B-deficiency to food consumption.

Investigators in this field seem almost unanimous that deprivation of vitamin B leads quickly to a reduced food intake, which, as the condition progresses, may gradually fall until, in the later stages, the animals are to all intents and purposes starving. The curves in Fig. 7 show typical records of weight, body temperature and food intake of young rats fed on the vitamin B-deficient ration. When their temperature falls the animals are virtually starving, with Calorie intakes as low as 3–4 per 100 g. as compared with about

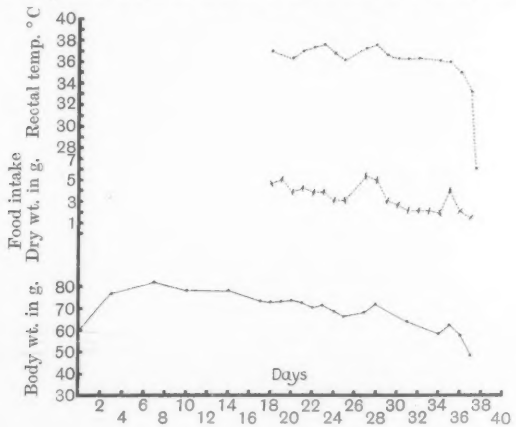

Fig. 7.
Rat 8623 ♂. Fed on vitamin B-deficient diet.
Rectal temp. •----------•
Food intake ¦----------¦
Body wt. •————•

30 Calories per 100 g. required for maintenance at this age. Obviously, therefore, no decision whether the fall in body temperature and metabolism in the last phase of the disorder is due specifically to the absence of vitamin B or not could be reached until the behaviour of animals on restricted food intake or deprived of food had been studied.

In a previous communication [Drummond, 1918] a study of the food consumption of rats on normal diets and on others deficient in vitamin B was reported, in which it was shown that soon after the administration of a diet deficient in vitamin B the food consumption fell to a level approximately that required to supply the calorific requirements for maintenance.

On this basis a series of rats were fed daily on a complete artificial diet

(that described on p. 1238), plus 5 % yeast extract, in amounts of the order that it was estimated would have been voluntarily consumed had the animals been given an ample supply of the vitamin B-deficient ration. Control groups provided with an unlimited supply of the complete diet and the deficient ration were also observed. The general result of these tests was that the groups fed on the restricted ration of the complete dietary (approximately 1 g. daily per 10 g. body-weight) maintained both body-weight and temperature, whilst occasional estimations of oxygen consumption yielded values within the normal range. Typical charts illustrating these results are shown in Fig. 8.

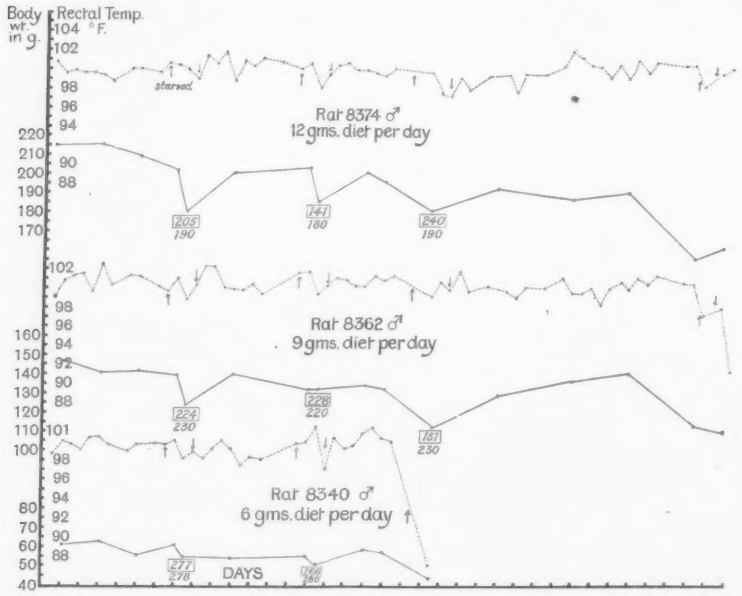

Fig. 8.

Rectal temp. •- - - -•- - - -•
Body weight •————•———— • of rats on complete diet but receiving a restricted quantity.
↑ Rats starved. ↓ Rats fed.
Oxygen consumptions in ☐ cc. per 100 g. per hr.; approximate normal value below.

In the smaller rats a decline in weight often occurred rather suddenly, and was accompanied by a rapid fall of temperature and followed by death.

The type of record obtained from the rats on the vitamin B-deficient diet is illustrated by the three pairs of curves in Fig. 9, each of which represents an animal of approximately the same initial weight as that of the corresponding animal in Fig. 8.

A general similarity of the records is immediately apparent on inspection, and it remains to study the response of animals to subsistence on water alone.

Typical charts of body-weights and temperatures of rats during inanition

are presented in Fig. 10, from which it can be seen that the latter seldom falls to any appreciable extent until shortly before death.

The fall of body temperature that occurs just before death as a result of deprivation of food is, as would be expected, accompanied by a fall in oxygen consumption, which, as in the case of the animals deprived of vitamin B, only becomes marked when the body temperature has dropped to about 33° or lower.

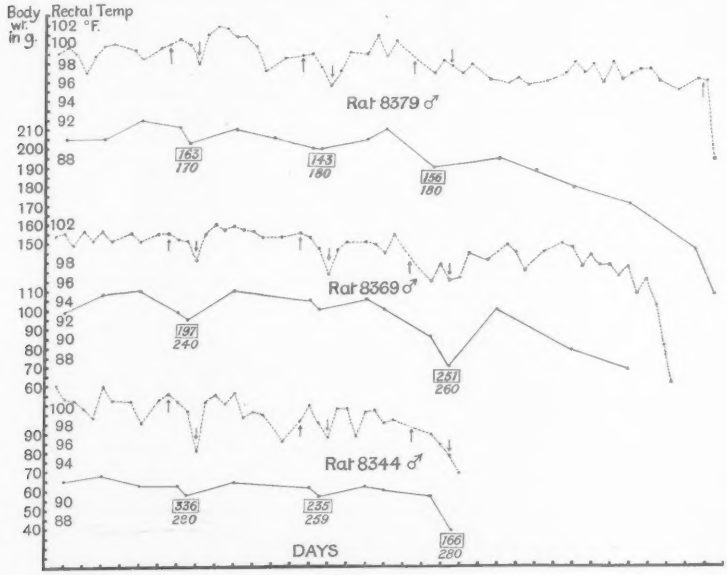

Fig. 9.

Rectal Temp. •-----•-----•
Body weight •————•———— of rats on B-deficient diet.
↑ Rats starved. ↓ Rats given food again.
Oxygen consumptions in ☐ cc. per 100 g. per hr.; approximate normal value below.

Table VII. *Oxygen consumption of rats during inanition determined at* 28°.

Weight g.	Day of experiment	Rectal temperature ° C.	Oxygen consumption	Approximate normal value (±20)
			cc. per 100 g. per hour	
62	3	31·0	142	280
64	4	30·0	90	280
64	6	30·5	84	280
75	4	35·0	363	260
73	4	35·2	274	260
70	5	36·4	238	265
92	6	38·3	271	250
113	7	30·0	76	240
116	8	37·5	323	240
119	8	34·4	187	235
125	7	28·5	40	225
130	8	34·4	185	225

By warming up the animals showing lowered metabolism, even when almost moribund, it was possible, as in the cases of vitamin B-deficiency described above, to increase their oxygen consumption to values approaching normal, and to effect a similar marked improvement of muscular tone and considerable increase in movement, but the improvement was only of short duration.

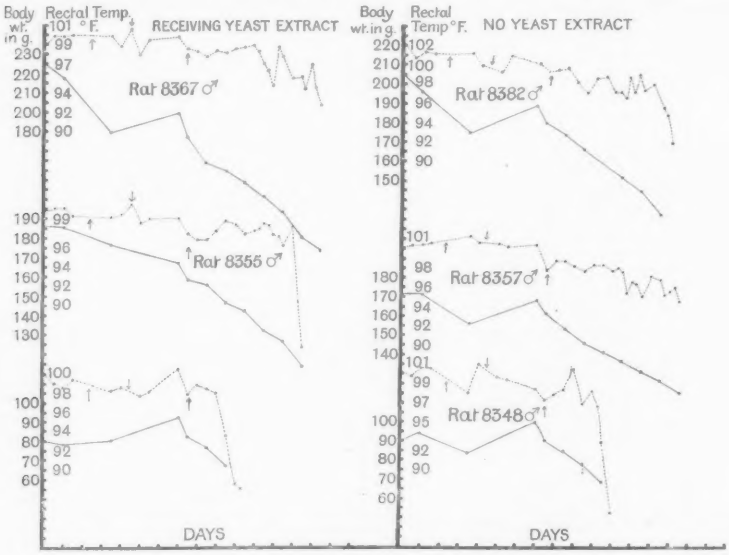

Fig. 10.

Rectal Temp. •– – –•– – –•
Body weight •————•————•
Rats during starvation.
↑ Rats starved. ↓ Rats fed.

Table VIII. *Effect of environmental temperature on oxygen consumption of rats in last stage of inanition.*

Weight g.	Days without food	Rectal temperature ° C.	O-consumption at 28°	Rectal temperature after warming in bath at 38° ° C.	O-consumption at 38°	Normal value
64	4	30·0	90	37·5	200	280
64	6	30·5	84	37·3	280	280
125	7	28·5	40	37·5	137	225
113	7	30·0	76	37·8	131	240

Relation of vitamin B-deficiency to inanition.

As several workers have pointed out [*e.g.* Walshe, 1918], total deprivation of food is actually a form of vitamin B-deficiency. Accordingly, a number of parallel experiments to those just described were carried out in which animals of similar weight were given daily from 0·5–1 g. of yeast extract as their sole

food together with ample supplies of water. The amount of utilisable food, from the standpoint of calories, supplied in this yeast extract was negligible.

In no essential did the behaviour of the animals in this group differ from those maintained on water alone (see Fig. 10). The provision of ample supplies of vitamin B neither prevented the final fall of body temperature, blood-sugar (see p. 1246) and metabolism nor prolonged life.

To our minds it seemed clear from these results that the lowered metabolism and body temperature observable during the last phase of the condition resulting from deficiency of vitamin B were merely indications that the animal was dying and that the immediate cause was general starvation. Before investigating the part played by the vitamin in inducing this state of affairs one important point remained to be examined. A number of investigators [cf. Funk and Schönborn, 1914; Shinoda, 1924, 1, 2; and others] have reported that vitamin B-deficiency brings about a condition of hyperglycaemia, and that, in pigeons at any rate, the blood sugar is high at the time that the characteristic convulsions appear. Unless the tissues had wholly or partly lost their power to oxidise blood-sugar, it was difficult to reconcile these claims with our own observations on the lowering of body temperature and metabolism. It was obviously necessary to make a study of the concentration of sugar in the blood of rats under the conditions of our experiments.

Blood-sugar level of rats on vitamin B-deficient diets.

Eggleton and Gross [1925] have described series of estimations of sugar in the blood of rats fed on the standard ration deficient in vitamin B used in this laboratory. They give the normal value of the blood-sugar level 24 hours after food as 0·09 % and the average after 40 days' subsistence on the deficient ration as 0·115 %. Later, in the final stage of the disorder, the concentration fell to the starvation level or lower. On these grounds they were, however, not convinced of the existence of a hyperglycaemia such as Funk and Schönborn had noted in pigeons.

In our experiments the estimations were made by the trustworthy method of Hagedorn and Jensen. A large series of determinations on normal rats kept without food for at least 6 hours gave the average value 0·082, the extreme values being 0·073 and 0·091.

The analysis of the blood of a number of rats deprived of vitamin B gave results which make clear two points. In the first place, there appears to be a definite hyperglycaemia in the rat during the greater part of the period it is kept on a ration deficient in vitamin B. Secondly, there is a very marked fall in the blood-sugar level during the final stage of decline when the body temperature and metabolism are also reduced. The following are typical results.

For purposes of comparison, estimations were made of the blood-sugar level in starving animals. Admittedly these are too few to be of real value, but, as far as they go, they are most suggestive in that they indicate a definite hyperglycaemia in what may be termed the middle phase of starvation followed

Table IX.

Weight g.	Rectal temperature ° C.	Days on B-deficient diet	% sugar in blood
58	34·0	29	0·105
58	34·2	31	0·083
45	34·4	31	0·115
57	34·1	32	0·116
47	34·5	56	0·109
65	34·3	41	0·154
70	33·9	41	0·140
44	33·5	22	0·078
73	35·5	45	0·156
62	35·0	29	0·146
Average for animals with body temperature above 33°			**0·120**
62	27·0	29	0·035
57	25·0	28	0·048
48	26·5	31	0·025
56	31·0	52	0·042
52	27·0	38	0·029
67	29·4	45	0·061
48	28·0	28	0·033
Average for animals with body temperature below 33°			**0·039**

by a rapid fall to very low values during the period when the body temperature drops. Morgulis and Edwards [1924] noted in starving dogs that the blood-sugar after a preliminary fall rose beyond normal values. The average values for starving rats of about 80 g. weight on the fourth day of the fast when their body temperatures were about 35° was found to be 0·125 %, whilst in the final period of decline with a body temperature of about 25° the blood-sugar level had fallen to an average of 0·023 %. Only a few estimations were made on the blood of rats whose body temperature had fallen below 30° as a result of deficiency of vitamin B, but had been restored to normal level by warming up in a chamber. The records of these experiments are given below.

Weight g.	Initial temperature ° C.	Final temperature ° C.	% blood-sugar
46	28·5	37·5	0·034
130	26·0	37·3	0·042
60	30·4	37·5	0·051
58	27·0	37·3	0·030
53	27·5	36·8	0·034
		Average	**0·038**

These figures seem to indicate that the temporary improvement in muscle tone and general activity that accompanies the artificial restoration of body temperature is probably not associated with an appreciable rise in the blood sugar level.

General discussion.

Before attempting a review of the results that have been set out in this paper, and stating the conclusions to which we think they lead, it would be well to draw attention to certain difficulties which render the task less easy than it at first appears.

Some of these arise from the uncertainty whether one or more than one

substance present in the yeast extracts employed as a source of vitamin B is responsible for the effects noted, whilst others appear as soon as the pitfalls that await one in attempting to correlate results obtained in the study of two species differing so widely from each other as the pigeon and the rat are appreciated.

Turning in the first place to the popular opinion that vitamin B, or some substance of similar distribution in natural foods, is essential for the production or functioning of the oxidative mechanism of the tissues, it must be apparent that our experience leads us completely to reject such view. To account satisfactorily for the opposing results of other workers, in particular those of Abderhalden and Hess, is not altogether easy. The experiments of the former investigator seem, however, open to criticism, if only on the grounds of the extraordinarily wide variability of his own figures. His employment of Ringer's fluid as the medium in which to conduct his experiments on the respiration of tissue suspensions seems unwise, and it is possible that some of the increases noted on adding various extracts supposed to contain vitamin B were actually due to a "buffering" effect.

Admittedly this would not explain all his results, which we must leave with the simple statement that we are entirely unable to confirm them on most essential points.

Passing reference may also be made to the claim of Abderhalden and Wertheimer [1923] that the tissues of "beri-beri" animals give the characteristic nitroprusside reaction for the sulphydryl grouping with diminished intensity. A series of estimations of glutathione made on normal and "beri-beri" tissues by the method of Tunnicliffe [1925] failed to reveal any differences. Our figures were as follows:

normal muscle 0·036 %;	normal liver 0·144 %
"beri-beri" muscle 0·038 %;	"beri-beri" liver 0·173 %

The researches of Hess and his collaborators fall into another category and must be criticised from an entirely different standpoint.

It is not easy to give an opinion on the significance of Hess's results on the administration of cyanides. As we have shown, the tissues from cyanide-poisoned animals showed no reduction of oxidative activity, owing, probably, to the great rapidity with which the salt in sub-lethal concentrations is detoxicated.

We are inclined to support Abderhalden's view that the condition of pigeons poisoned with cyanides is not related in any essential manner to that induced by a diet of polished rice, not for his reason that vitamin B will not effect the cure of a bird with cyanide poisoning, but rather on the grounds that it is unsafe to argue from a superficial similarity of symptoms, particularly the convulsions.

Our failure to obtain with methylene blue as an indicator results of the same general nature as Hess and his colleagues obtained with *m*-dinitrobenzene is, of course, no proof that there is any real contradiction, although

Lipschitz [1921] obtained with tissues and *m*-dinitrobenzene similar results to those Thunberg obtained with methylene blue.

We are indebted to our colleague, Mr R. K. Cannan, for the following expression of opinion as to the relation between the two groups of experiments.

"Whereas the 'methylene blue technique' has laid bare important catalytic properties of living cells *in vitro* and given quantitative expression to the specific activating powers of different cells [Quastel, 1926] its use in attempts to discriminate between the activity of normal and pathological tissue has not yet been accompanied by any great success. In the experiments reported in this paper there is no evidence—under the conditions of concentration of the tissue and reagents employed—of any difference between the rates at which normal and 'beri-beri' muscle activate succinate (as representative of Thunberg's 'metabolites') or the natural hydrogen donators of the tissue. A more detailed study of the kinetics of the reaction might have revealed minor distinctions, but it does not appear that we are at present in the position to interpret any but striking differences revealed by this technique.

"The observations of Hess remain to be explained, and there seems no present explanation for them. The use of *m*-dinitrobenzene as a hydrogen acceptor is not to be encouraged. The reaction *m*-dinitrobenzene—nitrophenylhydroxylamine is not reversible in the sense that the reduction of methylene blue is reversible and, moreover, does not instantaneously achieve equilibrium with the reductant of a truly reversible system. That is to say, following the argument of Cannan, Cohen and Clark [1926], we shall not expect *m*-dinitrobenzene to oxidise instantaneously the hydrogen donators activated by the tissue, so that the rates of reduction observed by Hess involved not only the velocity of hydrogen activation by the tissue but also the velocity of reaction of the added indicator. We are not in a position to disentangle these.

"Finally, since the *m*-dinitrobenzene system does not exhibit reversible potentials at an inert electrode, we have no measure of the region of reduction intensity which it may impose upon a system to which it is added. The discrepancy between the present results and those of Hess cannot therefore be accounted for by a difference in reduction potential attained, under anaerobic conditions, by normal and 'beri-beri' muscle betrayed by a *known* difference in potential of the H acceptors employed. Indeed, the reduction of *m*-dinitrobenzene probably involves more than one stage of hydrogen (or electron) transfer and is altogether unsuited as an indicator of biological oxidation-reduction. It were better, as Clark [1925] has emphasised, to discard all such indicators as are not electrochemically active and whose potentials have not been established, else the problem will be strewn with empirical data."

It is obvious, therefore, that in the face of our results we cannot subscribe to the view of Hess [1921]: "Wir erkennen somit als stoffliches Prinzip des sog. antineuritischen Vitaminfaktors die Bausteine oder die Muttersubstanz von Zellfermenten speziell der Atmungsfermente." At the same time, we must regard both the results of Dutcher [1918] on the catalase content of the tissues

in "beri-beri," and the observations of Rohr [1923] on the existence of a parallel between the oxidative activity and the vitamin B content of tissues with suspicion.

If, as a result of our observations on the rate of reduction of methylene blue and of utilisation of oxygen by muscle and liver, we accept the conclusion that the mechanisms for oxidations in these tissues are virtually unimpaired by withdrawal of vitamin B from the diet, it still remains for the recorded work on the respiratory metabolism of the whole animal to be considered. The originals of some of the earlier papers, in particular those of Raomino [1916] and Novarro [1920], have unfortunately not been available to us, and we are unable to express an opinion on their work and conclusions. Abderhalden [1921, 1, 2] reported greatly reduced gaseous exchange in pigeons nourished on the deficient diet of polished rice, and concluded from the influence of yeast extracts that the vitamin was necessary to prevent or cure a disorder of the cellular oxidations, although he did not subscribe to the more clearly defined views of Hess to which attention has already been drawn [Abderhalden, 1922; Abderhalden and Wertheimer, 1921, 1, 2, 1922]. Gulick [1922, 1924] observed that the basal metabolism of rats fed for 2 months on a vitamin B-deficient ration had fallen to 69 % of the normal value, but he also found a similar reduction in control animals which were given inadequate amounts of a complete diet. His conclusion that the fall was due to calorie starvation was supported by Anderson and Kulp's investigations [1922], as well as those of Farmer and Redenbaugh [1925].

Studies of the respiratory quotient in vitamin B-deficiency are numerous, and authorities are not in agreement whether the values obtained have any significance from the standpoint of the disorder.

Jansen and Mangkoewinoto [1920], Anderson and Kulp [1922], Mattill [1923, 1, 2], Fleming [1923], Okada, Sakurai and Kabishima [1923] seem to think that the values cannot in any way be regarded as characteristic of vitamin B-deficiency, whereas Raomino [1916] and Caridroit [1922] seem to attach more importance to the changes that may be observed. Actually, most investigators record values near 0·7 for animals in the advanced stage of vitamin B-deficiency; a fact that is not surprising when it is recalled that they are practically starving.

A more important point concerns the question whether there is or is not a reduction of general metabolism as a result of deprivation of vitamin B. It must be remembered that the investigations of Magnus Levy [1906], Lusk [1921] and Benedict [1919] have clearly demonstrated that there may be a fall in the level of basal metabolism amounting to as much as 20 or 30 % in conditions of under-nutrition associated with loss of body-weight. This can be ascribed to a reduction in the amount of what may be termed the "active metabolic tissue." Some reduction of the gaseous exchange might, therefore, be expected as a result of the loss of weight that results from vitamin B-deficiency.

The difficulties of obtaining satisfactory measurements of the basal metabolism of small animals such as rats are very great, unless one adopts the tedious procedure of waiting until the animals fall asleep in the chamber before measurements are made; forcibly to restrict the movements, as Farmer and Redenbaugh [1925] did by binding the wings and legs of pigeons with wide bandages, appears to us a most unsatisfactory attempt to overcome the difficulty. The errors that arise from variations in the muscular activity of the animals in the chamber, even when only small movements are visible, are, in our opinion, large and render difficult, if not dangerous, the interpretation of any but considerable differences in the oxygen consumption.

For that reason we cannot attach any significance to a large number of our results, or to an even larger number of those recorded in the literature, but, as we have remarked before, we obtained definitely low values from animals in the last stage of the vitamin B-deficiency.

Can these be regarded as supporting the view that the oxidative mechanisms are damaged?

A conclusive answer to this question is, in our opinion, provided by the experiments in which animals nearly moribund, with low body temperature and oxygen uptake, were restored to an almost normal metabolic activity by artificially warming their bodies. The recovery was associated with an extraordinary but transient improvement of muscular tone and increased activity and could scarcely have taken place if the oxidising mechanisms had been seriously inactivated.

There is, however, one more point adduced by the supporters of the "depressed oxidation" hypothesis to which attention must be given. That relates to the claim, many times advanced, that administration of extracts containing vitamin B will raise the body temperature of animals suffering from a deficiency of that substance.

If this were true it would certainly favour the view that the intracellular oxidations are stimulated by the administration of vitamin B, but the evidence is as yet far from convincing.

In our own experience with rats we have very occasionally observed a small and temporary rise of a degree or so in the body temperature of vitamin B-deficient animals after administration of yeast extract, but usually no response occurs. Such is not the case when sugar is given, for then the temperature rises slowly and steadily by several degrees and remains high for some hours. Positive results of this type recorded by other investigators who administered vitamin extracts alone to pigeons are, we think, possibly to be ascribed to another cause than a stimulation of intracellular oxidations. Almost all authorities are agreed that the pigeon, by the time it has reached the polyneuritic stage, although eating very little, may often carry in its crop considerable amounts of undigested food. It seems not improbable that the effect of the vitamin extracts, as far as heat production is concerned, is indirect in that their action is to increase the tone of the muscles of the alimentary

tract [cf. Gross, 1924], and to expel food from the crop. Further work is needed to clear this matter up satisfactorily.

Before dealing with the relation of the condition induced in rats by a deficiency of vitamin B to simple starvation, we might consider briefly the recurrent expression of opinion in the literature that the "beri-beri" animal cannot properly utilise carbohydrates. The evidence is conflicting and inconclusive. McCarrison [1919, 1, 2] supported the idea that digestion and assimilation of carbohydrates are imperfect in vitamin B-deficiency, and his views were supported by Magne and Simonnet [1922] who stated that the injection of glucose did not raise the R.Q. of a "beri-beri" animal, whereas it did in cases of inanition. They also state that the pigeon fed on vitamin B-free diet contains glycogen reserves which it cannot mobilise until the missing factor is supplied.

The idea that vitamin B-deficiency leads to imperfect digestion and absorption of carbohydrates, and perhaps to a lessened ability to mobilise and burn these substances, also lies behind the conclusions of Randoin and Simonnet [1923, 1924] who trace a quantitative relation between the quantity of vitamin B in the diet and the amount of carbohydrate absorbed.

Results entirely opposing those of Magne and Simonnet are reported by Mattill [1923, 1, 2] who found that the injection of cane sugar immediately raised the R.Q. of rats on a vitamin B-deficient diet from 0·75 to 0·9 or more; results which naturally led him to reject the idea that this malnourished organism was unable to oxidise carbohydrates. Support for his point of view is also provided by the experiments on carbohydrate absorption and utilisation of Eggleton and Gross [1925].

These discordant results really prolong the earlier controversy between Braddon and Cooper [1914] and Funk [1914], on the one hand, and Vedder [1918] on the other, regarding the existence of a relation between the amount of vitamin B and the carbohydrate metabolised.

A critical study of these and many other papers on the subject, together with our own work, has led us to doubt whether any such relationship exists [Reader and Drummond, 1926], but it is obvious that a definite decision must be postponed until more work has been carried out.

The idea that starvation is closely related to the conditions exhibited by animals deprived of vitamin B is by no means new, for it has been time and time again suggested by various authorities, although seldom with any clear definition.

Roche, to whose careful studies we have already referred [1925], remarks: "Si l'avitaminose ne saurait être uniquement réduite à l'inanition, il n'est cependant pas douteux qu'elle est, sinon partiellement conditionée, tant au moins accompagnée par une alimentation insuffisante," and concluded from his observations that the hypothermy certainly is a result of inanition and not specifically due to vitamin B-deficiency.

That the temperature must fall when the available food reserves are exhausted is obvious, and it seems that this is the most satisfactory explanation

of the hypothermy that is observed just before death whether from inanition or vitamin B-deficiency. Bierry [1919] has shown that it is coincident with a fall in blood sugar.

The evidence on which is based the opposite view of Weil and Mouriquand [1926] that vitamin B-deficiency and inanition are fundamentally unrelated is unconvincing.

Some reference must now be made to two abnormal conditions that are found in the organism as a result of deprivation of vitamin B, namely, certain lesions of the nerves and hypertrophy of the adrenal glands.

The former lesion is regarded by many as a characteristic and specific consequence of a deficiency of vitamin B. It is not intended here to review the extensive literature on this subject as it will form the subject of a paper by our colleague Dr Woollard, but it must be borne in mind that reliable authorities have more than once observed typical "beri-beri" in starving birds, particularly when life is prolonged beyond the usual span by administration of more water than the animal would voluntarily consume [Chamberlain, Blomburgh and Kilbourne, 1911; Chamberlain and Vedder, 1911; Eijkman and v. Hoogenhuyze, 1916].

The failure of other investigators to make the same observation [Frazer and Stanton, 1911; Funk, 1914; McCarrison, 1919, 2; Tscherkes, 1922, 1923, and others] seems likely to be due to the fact that their animals may have succumbed before there had been time for the disease to develop. In our own experiments on starving rats we observed two cases which presented an appearance closely resembling that of the "beri-beri" rat with characteristic posture of the hind limbs. Dr Woollard has found on careful histological examination that practically all our starved rats showed nerve lesions indistinguishable from those found in the vitamin B-deficient groups. This important observation is being followed up.

The second abnormality to which reference must be made, since it has been related by some authorities to the hyperglycaemia found in "beri-beri" animals, is the hypertrophy of the adrenals first noted by McCarrison [1919, 2]. This condition, standing out by contrast with the almost general atrophy of the other tissues [Funk and Douglas, 1914] is not characteristic of vitamin B-deficiency, unless starvation be regarded as a form of this disorder, because Vincent and Hollenberg [1920], McCarrison [1919, 2] and Findlay [1921, 1, 2] have recorded it in cases of inanition; a fact that has been confirmed by Dr Woollard. What this hypertrophy signifies is yet to be discovered. Funk [1919] regarded it as the cause of the hyperglycaemia he and Schönborn first noticed, whilst we have found that both are present in simple starvation. Riddle [1923] records hypertrophy of the adrenals and hyperglycaemia coincident in pigeons during ovulation. Cramer [1920] thought that the lowered temperature in vitamin B-deficiency might be due to interference with the heat-regulating function of the adrenal.

It is probable, however, that work along the lines indicated by the

researches of Cannon, McIver and Bliss [1923] will throw light on the matter. It will be remembered that these investigators demonstrated that any condition tending to cause a reduction of the level of sugar in the blood will lead to increased output of adrenaline.

To one other aspect of this many-sided problem we wish to call attention and we have reserved it until the last because we believe it may turn out to be the most important of them all. We refer to the studies of the relation of vitamin B to appetite made by Osborne and Mendel [1917], Karr [1920] and particularly Cowgill and his colleagues [1921, 1923, 1, 2, 1925, 1926]. The discovery of what appears to be a quantitative relation between the amount of vitamin B and the total food intake is of great importance in that it may provide the master key to the whole complicated problem.

Summary.

1. The oxidative activity of the muscle and liver tissue of rats and pigeons estimated either by direct measurements of oxygen consumption or by the rate of anaerobic reduction of methylene blue is not reduced as a result of the animals being deprived of vitamin B. This confirms the observations of Roche, but is in disagreement with those of Abderhalden, Hess and others.

2. The resting oxygen consumption of rats deprived of vitamin B remains at a normal level until the final phase of the condition of malnutrition induced by the deficiency. This phase, lasting 1 to 3 days, is characterised by a sharp fall of body-weight, rectal temperature and oxygen consumption.

3. Observations of food consumption indicate that a deficiency of vitamin B causes a progressive failure of appetite, leading eventually to almost complete abstention from food. The results of control experiments on animals deprived of food indicate that the fall of body temperature and oxygen consumption in the final phase of vitamin B-deficiency are probably the natural consequence of an exhaustion of reserve food materials.

4. That this view is probably correct, and that there is certainly no interference with the oxidative mechanisms of the tissues as a result of deprivation of vitamin B, is shown by the fact that animals exhibiting the premortal fall of body temperature and oxygen consumption will yield approximately normal values for the latter measurement after their temperature has been artificially raised to the normal range by warming in a bath. This recovery is, as would be expected, of very short duration.

5. Further evidence in support of this view is provided by studies of the concentration of reducing substance in the blood in rats in inanition and vitamin B-deficiency. In both conditions a state of hyperglycaemia develops —associated with a hypertrophy of the adrenal glands—but is followed by a sharp fall to values much below the normal level. The period during which this reduction occurs is that characterised by the fall of body temperature and oxygen consumption.

6. These results are discussed in the light of existing theories regarding the physiological action of vitamin B, and the conclusion is reached that, in the case of rats at any rate, the nutritive failure following a deficiency of the vitamin is virtually identical with that resulting from starvation.

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CLVII. RELATION BETWEEN VITAMIN B AND PROTEIN IN THE DIET OF GROWING RATS.

PHYSIOLOGICAL RÔLE OF VITAMIN B. PART II.

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(*Received October 21st, 1926.*)

In 1922 it was reported by Drummond, Crowden and Hill [1922] that cats and rats failed to grow at a normal rate when fed on dietaries containing 80–90 % of protein (caseinogen) but, to the best of our knowledge at that time, adequate as regards vitamins and salts. Furthermore, it was observed that in spite of the inhibition of growth the organs and tissues presented a normal appearance after several months on the high protein rations, and that the kidneys in particular exhibited no lesions of the type then rather generally believed to be caused by excessive nitrogen intake. Last year confirmation of these results was reported by the writers [1925].

Of possible explanations of the inability of the young animals to grow on the high protein dietaries, those based on the idea that a quantitative relation between certain dietary constituents may be necessary for normal nutrition seemed worthy of attention, particularly in view of the interesting observations of Hartwell on the relation between vitamin B and protein in the diet of pregnant and lactating rats [1924 and earlier papers].

It is uncertain what relationship exists between the symptoms described by Hartwell [1921] as being shown by young rats suckled by mothers fed on dietaries rich in proteins and the condition resembling beri-beri that can be produced by feeding these animals on a diet deficient in vitamin B. As we have remarked before [Drummond, Crowden and Hill, 1922], somewhat similar symptoms to those Hartwell described have been observed by us in very young rats being reared by mothers fed on a ration deficient in vitamin B. Later, Hartwell [1924, 1925] concluded from a continuation of her researches that for normal nutrition of the young it is necessary to maintain a *quantitative* relation between the level of protein and of vitamin B in the diet of the lactating mother. Assuming this relation to exist and also that it represents a relation between the vitamin and the metabolism of protein or its constituent molecules—for there is no evidence that in the absence of adequate amounts of vitamin B the digestion and absorption of protein is retarded or incomplete—it is of interest to enquire into the rôle of the former substance.

Drummond, Crowden and Hill [1922] suggested, on the grounds that it had been claimed that a quantitative relation exists between the vitamin B requirements of the organism and the total carbohydrate metabolised, that it might reasonably be argued that the vitamin would also be concerned in the metabolism of the glucogenic fragments of the protein molecules. Hartwell [1924] has pointed out, however, that this explanation does not serve to cover the results of a number of her experiments, and prefers to believe that "the rôle of vitamin B in the metabolism of protein is concerned with the nitrogenous part of the protein molecule or with protein *quâ* protein, and not with any fatty acid or carbohydrate residue." The following experiments were planned to throw further light on this question, in particular to determine whether the relationship traced by Hartwell serves to explain the failure of growth observed by us on high protein diets, and also whether any additional information could be obtained regarding other claims, namely those of Braddon and Cooper [1914], Funk [1914, 1919], Randoin and Simonnet [1923, 1924], that vitamin B is quantitatively related to the carbohydrate metabolised; of Tscherkes [1926] that the relation is between vitamin B and both protein and carbohydrate; and of Plimmer [1926] that the ratio between vitamin B and total food metabolised is normally a constant.

Experimental.

Two series of experiments were planned. In the first diets were employed of which the compositions were adjusted so as to give similar ratios between the amount of protein and the material supplying vitamin B, but to be of different calorific value. In the second series the proportion of protein was fixed whilst that of vitamin B was varied.

Series I. *Composition of Diets* (*parts*).

	A	B	C	D	E	F
Caseinogen	20	20	70	70	70	70
Starch	70	50	20	5	20	5
Hardened fat	0	18	0	18	0	18
Lemon juice	5	5	5	5	5	5
Salt mixture (McCollum) ...	5	5	5	5	5	5
Cod-liver oil	2	2	2	2	2	2
Yeast extract	4	4	16	16	4	4
Calories per 100 g. dry weight	360	480	360	480	360	480

Groups of young rats of approximately 50 g. weight were fed on each of these rations, close observation being made of their weight, food intake and general health whilst on the diet.

The growth curves of typical animals from each group are given in Fig. 1, from which it will be seen that the results of our previous work are generally confirmed, in that there is a failure to grow at a normal rate when the diet containing 70 % of protein is supplemented with only the same proportion (4 %) of yeast extract that suffices to produce good growth with 20 % of protein.

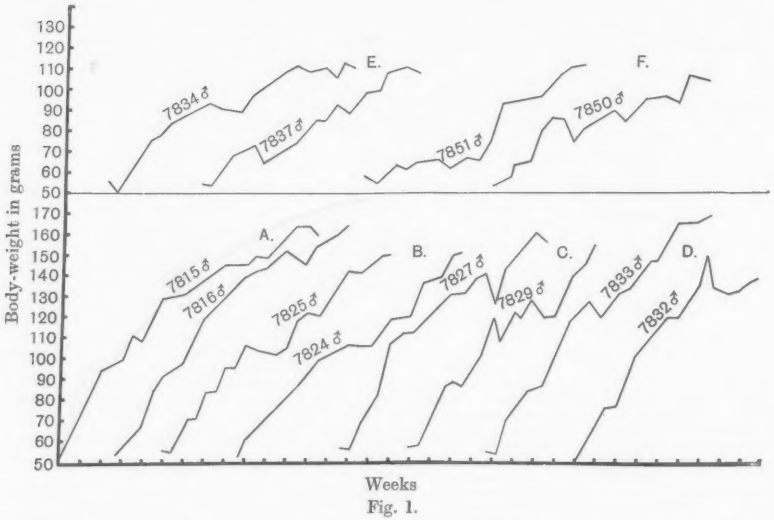

Fig. 1.

Observations of the amount of food consumed daily over periods of 14 days led to the following results being obtained (Table I).

Table I.

Group		Food consumption per 100 g. per day g.	Calorific intake per 100 g.	Approx. protein intake per day g.	Approx. intake of yeast extract g.	Average ratio Calories / Yeast	Average ratio Protein / Yeast	Growth
A	Males	5·4	19·4	1·1	0·22	88	4·9	Good
	Females	5·7	20·6	1·1	0·23			
B	Males	4·8	23·1	1·0	0·19	122	5·2	Good
	Females	6·0	28·8	1·2	0·24			
C	Males	7·0	25·4	4·9	1·12	22·6	4·3	Good
	Females	8·9	32·2	6·2	1·42			
D	Males	7·3	35·1	5·1	1·17	30·0	4·3	Good
	Females	7·8	37·5	5·5	1·25			
E	Males	8·0	28·8	5·6	0·32	88	18	Poor
	Females	10·4	36·4	7·3	0·41			
F	Males	10·5	50·5	7·3	0·42	120	17	Poor
	Females	11·0	52·9	7·7	0·44			

This analysis of the results of the estimations of food intake seems clearly to indicate a relation between the proportion of protein and the proportion of vitamin B in the diet as far as the effect on growth is concerned. On the other hand, there would seem to be no correlation between the total calories consumed and the vitamin B requirements, nor has any relationship been traced between the vitamin and the carbohydrate content of the diet, even when allowances are made in respect of the glucogenic value of the caseinogen.

The second series of experiments was made on young rats fed on rations containing the fixed proportion of 70 % of caseinogen, in which proportions

varying from 4 to 20 % of yeast extract were incorporated. Half the animals on each type of diet were given 15 % of fat (butter-fat) in their ration whilst the remainder were given no fat apart from a very small supplement of cod-liver oil to serve as a source of fat-soluble vitamins.

The growth curves of typical animals from each group and subgroup are reproduced in Fig. 2, from which it will be seen that a normal rate of growth is only attained when the 70 % of protein is supplemented with at least 16 parts of yeast extract, the addition of fat making no obvious difference. This is clearly illustrated by the curves in Fig. 3 in which are plotted the average body-weight of the rats in each group after a period of 10 weeks' feeding on the various diets against the percentage of yeast extract.

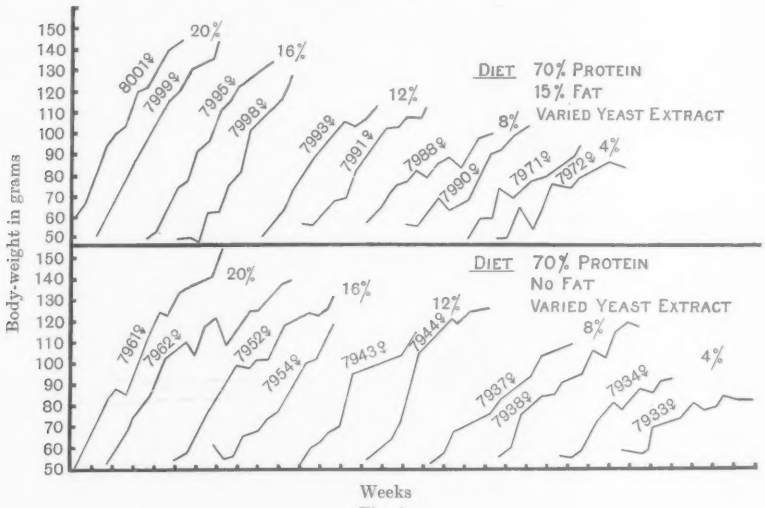

Fig. 2.

The ratios between the dietary constituents in these experiments are given in Table II.

Table II.

Diet	% protein	% yeast extract	Protein / Yeast ex.	Calories / Yeast ex.	Growth
1	70	4	17·5	90	Poor
2	70	8	8·6	45	Fair
3	70	12	5·8	30	Fairly good
4	70	16	4·4	22	Good, nearly normal
5	70	20	3·5	18	Normal
6	20	4	5·0	90	Normal

These results confirm those in Table I, in that there is no relation between total calories and vitamin B as far as correlation with growth is concerned, and that a normal rate of growth is not attained until the ratio between protein and yeast extract yields a value of about 5 or under.

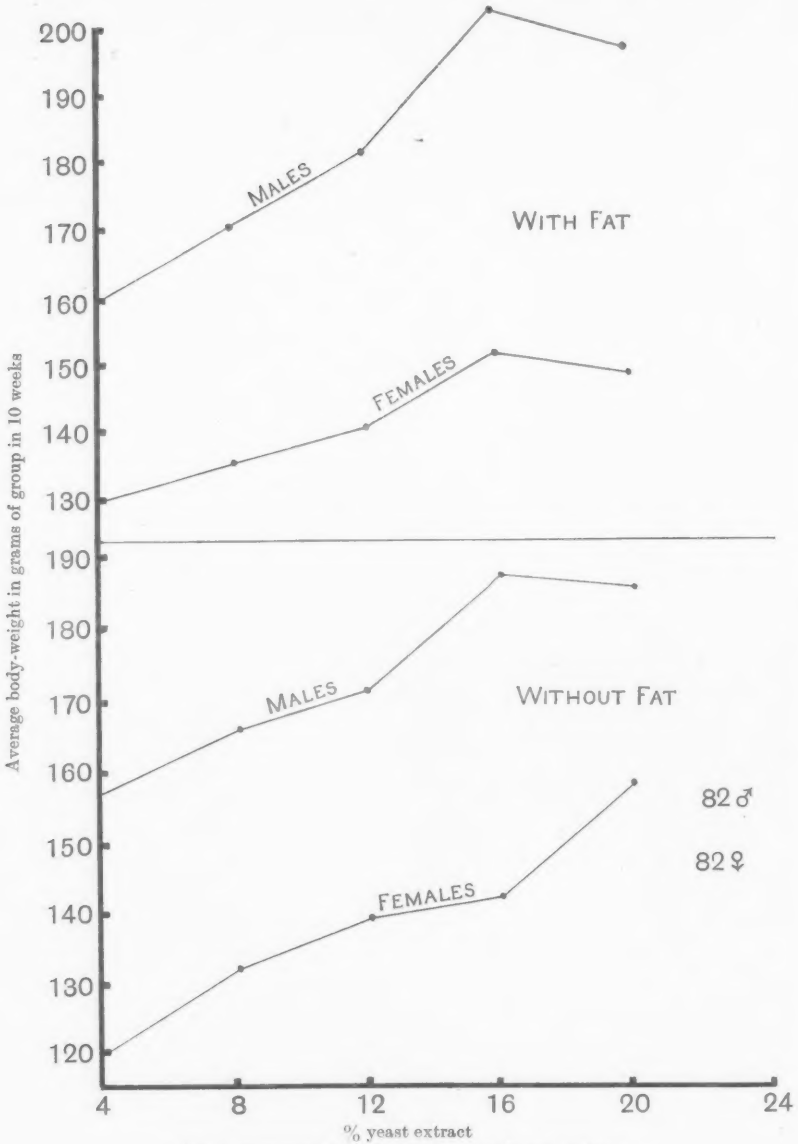

Fig. 3. Improved growth with increased yeast extract.

Effect on thyroid gland.

The possibility of a hypertrophy of thyroid tissue being induced on the high protein diets, particularly when the percentage of yeast extract is below that adequate for a normal rate of growth, was suggested and at the end of the experiments the glands were dissected out and weighed. In all groups, however, the ratio between the weight of thyroid and total body-weight was within the normal limits given by Donaldson [1924].

Table III.

Ratio $\frac{\text{Weight of thyroid}}{\text{Weight of body}} \times 1000$ on diets A—F.

Groups	A	B	C	D	E	F
	2·3	2·3	2·7	1·9	2·4	1·9
	1·6	2·0	1·8	1·6	2·3	3·0
	1·4	1·9	1·9	1·7	2·4	2·4
	2·3	2·1	2·3	1·6	2·0	2·5
	2·8	2·4	2·3	1·8	—	2·4
	1·9	2·0	—	1·8	—	—
	2·1	1·7	2·3	2·2	—	—
	2·2	2·1	2·5	1·5	—	—
Av.	2·1	2·1	2·2	1·8	2·4	2·4

Effect on kidneys.

As in the previous studies from this Laboratory the weights of the kidneys were determined after the animals had been 4 months on their respective diets. The results are given in Table IV.

The hypertrophy of the kidneys in groups E and F, fed on the diets rich in protein but relatively low in vitamin B, confirmed our previous observations. It was of the same order as that formerly detected by us and also by Jackson and Riggs [1926].

We wish again to emphasise the normal condition and histological picture of the kidneys of these rats and to reassert our belief that the excretion of relatively very large amounts of the nitrogenous end-products of metabolism is not in itself harmful to the renal tissues.

Scarcely any hypertrophy was apparent in the kidneys of the rats fed on the diets rich both in protein and vitamin B, a fact in agreement with the observed normal rate of growth of these animals.

Table IV.

Ratio $\frac{\text{Kidney-weight}}{\text{Body-weight}} \times 1000$.

Groups	A	B	C	D	E	F
	10·0	7·4	12·6	11·1	12·0	13·0
	10·4	7·8	13·5	9·1	14·5	14·0
	9·1	8·0	12·0	11·0	13·9	14·3
	8·6	9·6	13·3	8·0	15·0	14·8
	8·1	9·0	12·7	8·5	—	14·4
	10·2	14·0	11·3	10·0	—	—
	9·3	9·4	11·9	8·0	—	—
	10·1	14·0	—	10·0	—	—
Av.	9·4	9·9	12·4	9·4	13·9	14·1
	9·6		10·9		14·0	

Discussion.

From these results, as well as from the researches of Hartwell, it would seem established that a definite relation must exist between the intake of protein and of some factor in yeast, presumably the vitamin B, before normal nutrition is possible. Moreover, both investigations cast some doubt on the existence of a quantitative relation between the vitamin and the carbohydrate intake; a relation which, as has been pointed out, a number of investigators have claimed to have demonstrated.

Actually, however, a careful examination of the literature on this matter reveals a rather unsatisfactory state of affairs, in that many of the experimental results and the conclusions drawn from them seem unconvincing.

Against the claims of Braddon and Cooper [1914], Funk [1914, 1919], Randoin and Simonnet [1923, 1924], all of whom trace a relation between vitamin B and carbohydrate metabolism, can be set the work of Eijkman and van Hoogenhuyze [1916], Vedder [1918] and of Anderson and Kulp [1922], which lends no support to this view.

Much of this work was concerned with determining the time of onset of the so-called "polyneuritic" symptoms in pigeons fed on different types of diets and seems on critical examination to be lacking in precision and adequate control.

By employing a similar method, Tscherkes [1926] believes he has traced a relation between the requirements of the organism for vitamin B and the intake of carbohydrates and protein, but that the vitamin is unrelated to the fat of the diet. Naturally, many of the points raised by this work are bound up with the question whether the growth-promoting factor in yeast (vitamin B) is identical or not with that protecting the organism against the so-called beri-beri.

In view of the fact that the evidence on this matter is still far from complete and that the assumption that only one factor is concerned is not at variance with any recognised facts, it seems wiser in the present state of our knowledge to accept the simpler hypothesis. Assuming, then, that only one substance is concerned, it is not possible with our results in mind to subscribe to the opinion that the vitamin B is quantitatively related, as far as growth in the rat is concerned, either to the carbohydrate intake or to the total calories consumed.

What part the vitamin plays in protein metabolism remains a matter for further investigation, but at first sight it would seem that Hartwell is right in believing that it is concerned with the fate of the proteins or their constituent units as a whole rather than with the metabolism of the nitrogen-free residue of the amino-acid molecules, as was at one time tentatively suggested.

SUMMARY.

1. Further confirmation has been obtained that diets consisting largely of protein (caseinogen) may be inadequate for the growth requirements of young rats.

2. This inadequacy may be made good by raising the proportion of yeast extract (vitamin B). Normal rates of growth were obtained on dietaries in which the ratio of protein to yeast extract has a value of 5 or under.

3. The results of these experiments seem to confirm the views of Hartwell and of Tscherkes that vitamin B is related quantitatively to the amount of protein in the diet.

They do not lend support to the opinions that the vitamin stands in such relation to the amount of carbohydrate ingested or metabolised or to the total calories consumed, at any rate as far as the effect on growth is concerned.

4. Additional evidence has been obtained of the hypertrophy of the kidneys in animals fed on diets consisting largely of proteins. This hypertrophy is only marked when the composition of the diet is such that the animal does not grow at a normal rate. As pointed out in previous publications the excretion of large quantities of nitrogenous end-products of metabolism does not cause damage to the kidneys.

We wish to thank the Medical Research Council for a Grant from which the expenses of this research were defrayed.

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CLVIII. STUDIES ON THE INTERACTION OF AMINO-COMPOUNDS AND CARBOHYDRATES.

III. THE ACTION OF NITROUS ACID ON CERTAIN NITROGENOUS SUGAR DERIVATIVES AND RELATED COMPOUNDS.

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(*Received October 26th, 1926.*)

In continuation of our investigations on the behaviour of glucose ureide [Hynd, 1926] it was necessary to study the action of nitrous acid on the compound, even though Schoorl [1903] has reported the formation of glucose from the ureide as the result of the interaction of nitrous anhydride.

From the results of preliminary experiments carried out by one of us (A. H.) over a year ago, it seemed that, under conditions similar to those of Van Slyke's technique for the determination of NH_2-groups in amino-acids, glucose ureide failed to react with nitrous acid, but on the addition of a mineral acid fully half of the total nitrogen was liberated rapidly. As this result was in agreement with the findings of Werner [1917] regarding the behaviour of urea towards nitrous acid, but contrary to those of other workers, a closer study of the reaction seemed desirable. Consequently an investigation of the behaviour of urea and related compounds towards nitrous acid under various conditions was planned and experiments were in progress when Plimmer [1925] published the results of experiments covering this same field, though undertaken for a somewhat different purpose.

Reference to the experimental part will show that our results for urea are in close agreement with those reported by Plimmer, and as a similar agreement was obtained in the case of urethane, guanidine carbonate, creatine, and creatinine, our results for these compounds need not be given in detail. Accordingly, it is only necessary to report fully the nitrogenous sugar derivatives investigated, though reference will be made to the other results in the course of the discussion.

Experimental.

All the experiments were carried out in Van Slyke's micro-apparatus, the usual method being followed. In those cases where mineral acid was required, 40 % sulphuric acid was used and was introduced by means of the side burette. The reaction chamber was shaken continuously in experiments of short dura-

tion, but during the first and last half-hours only, when the reaction was continued for a long period.

The nitrogen evolved was measured, and by making use of Van Slyke's table [1912] was converted into milligrams; all the results are expressed as mg. nitrogen per 100 cc. of solution. The concentrations of the solutions employed were checked either by carrying out total nitrogen determinations by Kjeldahl's method or by the rotations in the case of the optically active compounds.

Compound	Medium	Time	Temp. °C.	Amino-N found mg.	% of total N
Urea	20 % acetic acid	½ hr.	19	12·7	5·53
	,,	1 ,,	16	47·6	20·6
	,,	1½ hrs.	16	81·2	35·1
	,,	2½ ,,	16	177·4	66·8
	,,	22 ,,	16	232·0	100·6
	,, +1 % H_2SO_4	5 mins.	20	52·7	22·8
	,, +3 % ,,	5 ,,	20	213·2	92·3
	,, +6 % ,,	5 ,,	21	183·4	79·5
	,,	5 ,,	20	180·8	78·3
	,,	5 ,,	20	174·9	75·8
	,, +10 % ,,	10 ,,	19	172·7	74·5
	,,	15 ,,	19	176·6	76·5
	,,	30 ,,	18	230·7	99·9
	,,	30 ,,	19	234·1	101·4
Urethane	20 % acetic acid	22 hrs.	15	11·4	6·28
	,, +8 % H_2SO_4	22 ,,	14	188·3	103·9
Guanidine carbonate	20 % acetic acid	2 ,,	16	0	0
	,, +12 % H_2SO_4	23 ,,	14	135·8	102·3
	,, +6 % ,,	21 ,,	15	136·3	102·7
Guanidine nitrite	20 % acetic acid	24 ,,	14	0	0
	,, +6 % H_2SO_4	21 ,,	15	83·2	93·1
Creatine	20 % acetic acid +6 % H_2SO_4	21 ,,	14	52·8	67·9
Creatinine	20 % acetic acid	1 ,,	17	153·2	36·1
	,,	2 ,,	18	159·6	37·6
	,,	18 ,,	14	166·4	39·2
	,, +3 % H_2SO_4	23 ,,	16	150·9	35·5
Creatine methyl ester hydrochloride	20 % acetic acid	2 ,,	18	79·8	45·5
	,, +3 % H_2SO_4	17 ,,	15	114·2	65·
"Creatine methyl ester"	20 % acetic acid	2 ,,	16	55·1	41·3
Glycine	20 % acetic acid	10 mins.	16	41·22	100·3
	,, +6 % H_2SO_4	10 ,,	15	41·36	100·7
Glucosimine	20 % acetic acid	½ hr.	19	158·7	81·35
	,,	1 ,,	19	176·0	87·1
	,,	1¾ hrs.	19	181·2	92·9
	,,	2 ,,	19	196·8	101·2
	,, +6 % H_2SO_4	10 mins.	19	168·9	86·6
	,,	½ hr.	19	194·0	99·5
Glucosamine	20 % acetic acid	1 ,,	19	18·0	25·0
	,,	18 hrs.	19	74·3	103·4
Glucosamine hydrochloride	20 % acetic acid	½ hr.	19	137·36	95
	,,	¾ ,,	19	144·0	100·3
	,, +5 % H_2SO_4	½ ,,	19	137·3	95·6
	,,	2 hrs.	19	147·6	102·8
Glucose ureide	20 % acetic acid	20 mins.	19	4·58	1·8
	,,	20 ,,	19	0	0
	,,	24 hrs.	19	118·3	46·36
	,, +5 % H_2SO_4	5 mins.	19	153·8	60·2
	,,	½ hr.	19	167·0	66·5
	,,	2½ hrs.	19	169·6	67·6
	,,	24 ,,	19	165·5	65·9
	,, +10 % H_2SO_4	15 mins.	19	151·26	60·2
	,,	½ hr.	19	163·1	63·8
	,,	1 ,,	19	167·0	66·5
	,,	2¾ hrs.	50	218·5	87·0

Compound	Medium	Time	Temp. °C.	Amino-N found mg.	% of total N
Glucose ureide	,,	3 ,,	50	232·3	92·5
	,,	4 ,,	50	252·5	100·5
Glucose ureide-urea	20 % acetic acid	20 mins.	19	43·0	7·16
	,,	½ hr.	19	64·1	10·68
	,,	1 hr.	19	110·3	18·3
	,,	2 hrs.	19	162·1	26·98
	,,	20 ,,	19	506·7	84·2
	,, +5 % H_2SO_4	½ hr.	19	422·2	86·4
	,,	2 hrs.	19	427·8	87·5
	,,	24 ,,	19	430·0	87·9
	,,	2 ,,	50	427·8	87·5
	,,	5¼ ,,	50	478·5	97·9
	,,	7 ,,	50	490·1	100·3
Lactose ureide	20 % acetic acid	½ hr.	19	0	0
	,,	18 hrs.	19	52·98	47·3
	,, +5 % H_2SO_4	20 mins.	19	65·36	58·7
	,,	½ hr.	19	72·06	63·9
	,,	2 hrs.	19	72·98	64·7
	,,	19 ,,	19	78·94	70·05
	,,	4 ,,	50	113·9	101·03
	,,	4 ,,	50	116·5	103·3
"Glucosamine + urea product"	20 % acetic acid	24 ,,	18	0	0
	,, +5 % H_2SO_4	24 ,,	18	0	0
	,,	10 ,,	50	0	0

Discussion of results.

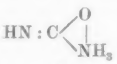
With the one exception of the product obtained in an attempt to condense urea with glucosamine, all the compounds examined react with nitrous acid under suitable conditions. But the compounds are divided, though perhaps not sharply, into three groups according as the reaction proceeds (1) rapidly, or (2) slowly in the presence of a weak organic acid such as acetic acid, or (3) requires the presence of a strong mineral acid, such as hydrochloric acid or sulphuric acid, to bring about a significant evolution of nitrogen gas. Typical examples in the first group are glycine and glucosimine; in the second group, urea, glucose ureide, glucosamine and creatinine; and in the third group, urethane, guanidine and creatine.

To account for these differences it is evident that the nitrogen atom or atoms involved must be linked in various ways, which it should be possible to indicate by means of structural formulae. Although guanidine, on this basis of structure, may be regarded as the parent compound, it will be advisable to deal first with urea, as its structure has been more fully investigated [Werner, 1923].

Urea. Werner [1917] claims that urea is not attacked by pure nitrous acid alone or even when a second very weak acid is present. Consequently the practically complete, though slow, decomposition of urea by nitrous acid in the presence of acetic acid here recorded may seem at first sight contrary to this statement, but it is to be noted that the experimental conditions were widely different. In the first place, the concentrations of both acetic acid and nitrous acid in our experiments were high, and secondly, conditions for the partial decomposition of the nitrous acid, according to the equation

$$3HNO_2 \longrightarrow HNO_3 + 2NO + 2H_2O,$$

are favourable during Van Slyke's process, especially when the reaction is allowed to proceed for a long period such as 24 hours at 20°. However, as Werner's experiments lasted from 1 to 3 days at 13°, neither the temperature nor the duration of the reaction appears to be an important factor, though both Dunn and Schmidt [1922] and also Wilson [1923] have observed the rate of deamination of certain amino-compounds to be affected by temperature. It seems reasonable to suppose that in our experiments the hydrogen ion concentration of the medium became sufficiently high slowly to convert urea from its inactive cyclic structure into its reactive open form. Further support is given to this view by the fact that the addition of a strong acid rapidly brought about complete deamination. Accordingly, urea in a neutral medium exists as a ring compound, represented by I or III, either of which becomes converted into the *iso*-carbamide structure II on increase of hydrogen ion concentration. The free amino-group is then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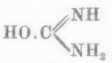
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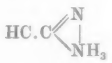
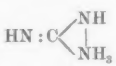

III

attacked by nitrous acid, nitrogen being liberated, and the residual cyanic acid, as explained by Werner, yields a second atom of nitrogen.

Structure I is that proposed by Werner, but we are inclined to favour III, for, as will be clear from what follows, its adoption brings urea, guanidine and creatine better into line.

Guanidine. We find that guanidine, in presence of acetic acid, reacts with nitrous acid only very slightly if at all, while in presence of the necessary amount of mineral acid practically the whole of the nitrogen is evolved after 24 hours. These results agree with the experimental findings of Plimmer [1925], but are contrary to the observations of Krall [1915], who states that nitrous acid reacts slowly and liberates nitrogen corresponding with no more than one of the three nitrogen atoms, even when excess of a strong acid is present. Consequently, this worker suggested that guanidine in solution as the free base is a mixture, in approximately equal proportions, of the isomerides IV and V, formulated below, strong acid increasing the proportion of the latter, whereas alkali hydroxides increase the proportion of the former. This view is also supported by the recent study of the hydrolysis of guanidine carried out by Bell [1926]. Plimmer, on the other hand, considers guanidine to have formula IV, changing to VI in the presence of mineral acid, which he holds explains the liberation of two-thirds of its nitrogen in the presence of hydrochloric acid.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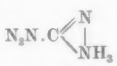
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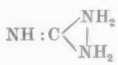
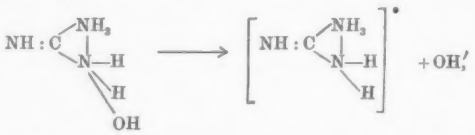

VI

Taking cognisance of the fact that all attempts to prepare a dihydro-

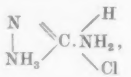
chloride from guanidine have been unsuccessful, we take the view that guanidine is best represented by the structure:



which, as indicated, would dissociate as a strong base, and form stable salts even with weak acids. Such salts would be hydrolytically dissociated and accordingly their solutions would have an alkaline reaction. This is in agreement with the well-known fact that guanidine carbonate $(CH_5N_3)_2 . H_2CO_3$ is comparable with sodium carbonate and forms strongly alkaline solutions. Further, we found that a solution of guanidine nitrite can be obtained by mixing equivalent solutions of guanidine carbonate and barium nitrite and then removing the precipitated barium carbonate by filtration. No evolution of nitrogen was observed from a $M/20$ guanidine nitrite solution thus prepared which had p_H 9 approximately. This stability is consistent with the isolation by Thiele [1893] of nitrosoguanidine, to which he assigned the formula

$$H_2N.C(:NH).NO.$$

Accordingly, owing to the stability and strongly basic nature of the guanidine ion, no reaction with nitrous acid takes place in the presence of acetic acid, that is, decomposition does not occur so long as the hydrogen ion concentration remains low. On increasing the hydrogen ion concentration, as by the addition of hydrochloric acid, evolution of nitrogen ensues. During this process, there is probably conversion to the structure

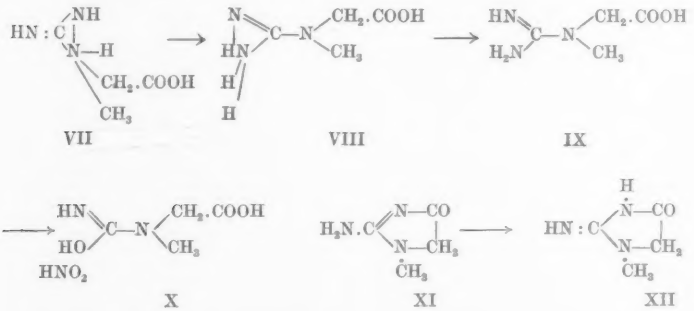


the free amino-group then reacting normally with nitrous acid, nitrogen being liberated and the rest of the molecule appearing as structure III above, which in the presence of mineral acid would pass into II. Consequently, urea is to be regarded as the first intermediate product in the decomposition of guanidine by nitrous acid. The subsequent stages being those already indicated for urea, the whole of the nitrogen is liberated, a result in accordance with the behaviour of arginine under similar conditions (Plimmer). It is to be noted, however, that it would be impossible to detect the intermediate formation of urea, as, under the conditions necessary for the first stage of the reaction which is a slow one, the urea would be decomposed as fast as it was formed.

The conversion of form IV into V is not regarded by us as a simple tautomeric change produced by the wandering of a hydrogen atom from the NH in the ring to the NH outside the ring. The initial step seems to involve rupture of the bond between the two ring nitrogen atoms. Redistribution of the hydrogen

atoms then occurs with the formation of a ring structure, in which the nitrogen atom outside the original ring structure now takes part, while that one of the original ring-nitrogen atoms, which is now outside, carries the additive salt molecule. This gives a satisfactory explanation of the existence of mono-acid salts of guanidine. However, probably owing to the presence of the double bond, the ring structure in V is not so stable as that in IV and can be ruptured by even a weak acid, so that in solution a salt of guanidine with a strong acid may exist as represented in form VI.

Creatine. From the fact that both Volhard and Horbaczewski used sarcosine as the starting material for their syntheses of creatine, it is clear that the methyl and the carboxy-methyl groups must be linked to the same nitrogen atom. Further, as the compound reacts towards nitrous acid in the same way as guanidine, it appears to us to have structure VII, in solutions of low hydrogen ion concentration, which, as explained in the case of guanidine, tautomerises to the form VIII when the hydrogen ion concentration is sufficiently high. In solution conversion to IX would occur, and consequently on reaction with nitrous acid one nitrogen atom would be evolved with production of an intermediate X similar in structure to the acid amides (Plimmer), so that a second nitrogen atom would be liberated.



VII VIII IX

X XI XII

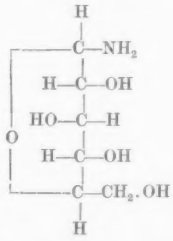
Creatinine. Further action of hydrochloric acid on compound VIII brings about rupture of the bond between the nitrogen atoms, and dehydration occurs, probably without the intermediate formation of IX, so that creatinine results with structure XI. Consequently we find, as previously reported by Plimmer [1925] and also by Wilson [1923], that creatinine reacts with nitrous acid in the presence of acetic acid to lose one of the three nitrogen atoms. The same result is obtained in presence of hydrochloric acid unless excessive amounts are used. Plimmer explains the effect of mineral acid in decreasing the amount of nitrogen evolved as due to a change to the alternative structure XII. However, as a similar retarding influence was noticed by us in the case of urea when excessive amounts of sulphuric acid were used, the correct explanation is not clear. In this connection it may be noted that no difference

was produced in the result of the decomposition of glycine by nitrous acid in the presence of acetic acid by adding excess of sulphuric acid.

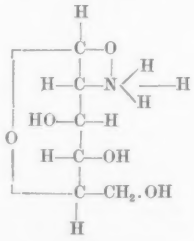
Creatine methyl ester hydrochloride. This compound has been claimed by Kapfhammer [1925] to be a derivative of creatinine, but, as 65·1 % of the total nitrogen is evolved on reaction with nitrous acid in the presence of sulphuric acid, the compound was correctly described by Dox and Yoder [1922] as the hydrochloride of creatine methyl ester. However, as 41·3 % of the total nitrogen is liberated from the compound when its solution is shaken with a slight excess of silver carbonate and then decomposed by nitrous acid in the presence of acetic acid, removal of the salt molecule evidently involves destruction of the ester with elimination of methyl alcohol and the resulting formation of creatinine. This would seem to give a satisfactory explanation of Kapfhammer's observations.

Glucosimine and glucosamine. The former compound, like glycine, was found to yield the same results with acetic and sulphuric acids. In both cases, rapid decomposition of glucosimine with nitrous acid occurs, as noted by Levene [1916], and also by Schmuck [1923].

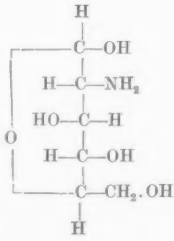
This behaviour forms a marked contrast to that of glucosamine, which required 18 hours for complete decomposition in the presence of acetic acid. This supports the view that glucosimine should be formulated with a free amino-group, as in XIII, while glucosamine, as it reacts in a manner similar to urea, must contain in its molecule, as previously suggested by Irvine and Hynd [1913], the modified betaine ring structure, as in XIV, which in the presence of mineral acid changes to XV; accordingly the compound yields the total amount of nitrogen in about half an hour.



XIII



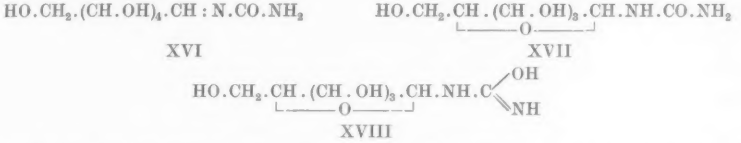
XIV



XV

Ureides. (1) *Glucose ureide.* The formula XVI originally assigned to this compound by Schoorl [1903] requires modification in two respects. Alteration of the glucose portion to an oxidic ring structure [cf. Helferich and Kosche, 1926], probably amylene oxide, as in XVII, gives a better explanation of the results obtained on acetylation [Schoorl, 1903]. With regard to the urea portion, no free amino-group is present, as with nitrous acid in the presence of acetic acid only slow evolution of nitrogen amounting to about 50 % of the total nitrogen was obtained. Consequently a ring structure like that in urea is

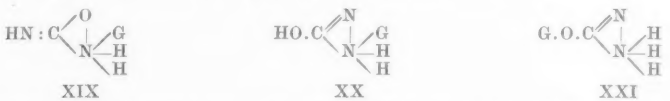
indicated, as the alternative form XVIII, on the analogy of the behaviour of urethane and the acid amides, would not be expected to yield any nitrogen in the presence of acetic acid.



XVI XVII

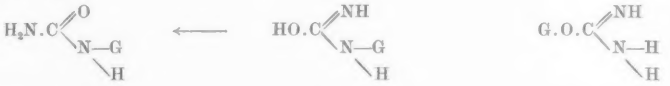
XVIII

With regard to ring structures three possibilities exist, which, if we write G for the glucose residue, may be represented as follows:



XIX XX XXI

In the presence of acetic acid these would exist as XXII, XXIII, and XXIV respectively:



XXII XXIII XXIV

XXIII then passing into XXII. With the data at present available it is impossible to decide which of the above ring structures should be adopted for the compound, but from the results of another series of experiments which are all but complete, it is hoped that it will be possible to make a definite choice.

The fact that 50 % of the total nitrogen is readily obtained by reaction with nitrous acid either in the presence of acetic acid or sulphuric acid, while even in the presence of mineral acid the reaction requires to be continued for several hours at 50° before 100 % of nitrogen is liberated, suggested that it might be possible to isolate an intermediate product and thereby furnish experimental evidence in support of Werner's views on the structure of urea and its derivatives. Various experiments were carried out with this end in view, using either silver nitrite and hydrochloric acid or barium nitrite and sulphuric acid to generate the necessary nitrous acid, but all ended in failure. The only crystalline product isolated was a small proportion of unchanged glucose ureide (mixed M.P. 203°). The main organic product obtained was an extremely hygroscopic syrup, which could not be obtained crystalline. It reacted like glucose.

(2) *Glucose ureide-urea.* The behaviour of this compound towards nitrous acid supported the view that the substance is a simple additive compound. No further comment is required.

(3) *Lactose ureide.* This product obtained according to the method described for glucose ureide [Hynd, 1926] has the same melting-point and

specific rotation as given by Schoorl, and reacts with nitrous acid in a similar manner to glucose ureide.

(4) Product obtained from glucosamine-urea. The preparation and properties of this compound, which so far has not been fully examined, will be described later. It may be mentioned, however, that it does not react with nitrous acid under any conditions—a rather unexpected result in view of the character of the starting materials used in its preparation.

Conclusions.

1. The so-called amino-compounds can be subdivided into three groups according to their behaviour towards nitrous acid.

2. Glycine and glucosimine are typical examples of the group in which nitrogen is liberated readily on reaction with nitrous acid in the presence of a weak acid.

3. Urea, glucose ureide and glucosamine are representative of a group in which reaction with nitrous acid proceeds slowly in the presence of a weak acid but rapidly in the presence of a strong acid.

4. Urethane, guanidine and creatine are typical of those amino-compounds which on treatment with nitrous acid require the presence of a strong acid before any appreciable evolution of nitrogen takes place.

5. Structural formulae are assigned to each of the compounds investigated. These are in most cases slight modifications of those generally accepted, and account for the difference in behaviour towards nitrous acid observed. They are also, at the same time, consistent with the other properties of the respective compounds.

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CLXI. GROWTH AND REPRODUCTION ON SYNTHETIC DIETS. PART I.

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THE use of synthetic diets in nutrition experiments occupies a prominent place in modern research. For many years American workers have used what may be termed "semi-synthetic" diets in which such constituents as rolled oats, milk powder, etc., have formed a large bulk of the food, but experiments with *true* synthetic diets are not so numerous. It is of great importance to the nutrition worker to have a good standard synthetic diet, but at the present time such a food is not available. Various workers claim normal growth for animals on synthetic diets, but no control curve is given [*e.g.* Reader and Drummond, 1925]. In many instances the growth curve of Donaldson [1924] is taken as the standard, and it is quite true that rats on synthetic diets frequently show better growth than this, though not nearly so good as on a mixed diet of natural foods. The recent work of Osborne and Mendel [1926] shows that an improvement in the diet of the rat leads to a better rate of growth. These workers also found that the best growth was obtained on synthetic diets plus small additions of "natural foods," *e.g.* lettuce or liver. This obviously suggests that our synthetic diets are by no means perfect, and research work has yet to discover a synthetic diet which will produce as good growth as does the ordinary mixed diet of natural foods. Sherman and Campbell [1924] found that increasing the proportion of milk in an already adequate diet led to improved nutrition. Similar progress in true synthetic diets is badly needed. Mitchell [1922] added agar-agar to a synthetic diet and found that this led to the females breeding and rearing young, when they had previously failed. She suggests that bulk in the diet is a factor of importance. Previous work of the author [Hartwell, 1925, 2] also shows that the usual synthetic diets failed in this respect, and a method was described for overcoming this difficulty in another way. It seems useless to discuss the amounts of the various vitamins necessary for normal growth when we are using a diet which in itself only permits submaximal growth. Therefore, at the present time, it appears to be of vital importance for us to have a true synthetic diet which will provide for growth equal to that produced on ordinary mixed food. The experiments described in this paper were started in the hope of obtaining an improved synthetic diet.

Experimental.

Animals and method.

Piebald rats bred in the laboratory were used. For the majority of the experiments 12 animals, 6♀ and 6♂ were taken; they were kept in a large cage together so that breeding should proceed normally. The synthetic diet was started at the age of 23 days, food and water being given *ad lib.* The rats were weighed daily for 4 weeks, after which the weighings were omitted on Saturdays and Sundays. Later, when the gain was less, the animals were weighed weekly. Pregnant does were removed from the large cage a few days before the birth of the litter. The young and the mother were weighed every day. After suckling her babies for 21 days, the doe was returned to the big cage (except in one case where the young were not very fit and could not eat for themselves at the usual time).

Diets.

All diets were prepared according to the "cooked" method [Hartwell, 1925, 2].

Diets of the following composition were given:

	Diet					
	1	2	3	4	5	6
Caseinogen (purified; free from vitamins A and B)	20 g.	20 g.	20 g.	—	10 g.	10 g.
Food casein [Hartwell, 1922]	—	—	—	20 g.	—	—
Edestin* (94·36 % protein) ...	—	—	—	—	10 g.	10 g.
Gluten [Hartwell, 1925, 1] ...	—	—	—	4 g.	—	—
Starch (potato, pure)	64 g.	64 g.	64 g.	60 g.	64 g.	64 g.
Butter	12 g.	12 g.†	12 g.	—	12 g.	12 g.†
		or	or			or
Cod-liver oil	—	4 cc.	4 cc. ‡	4 cc.	—	4 cc.
Cotton-seed oil	—	10 cc.	10 cc.	10 cc.	—	10 cc.
Salt mixture [Hartwell, 1922, 1]	4 g.	4 g.	4 g.	4 g.	4 g.	4 g.
Marmite	2 g.	2 g.	5 g.	2 g.	2 g.	2 g.
Extract from marmite ... (1 cc. = 1 g. marmite)	—	3 cc.	—	3 cc.	—	3 cc.
Water	300 cc.	297 cc.	300 cc.	297 cc.	300 cc.	297 cc.

* The edestin used in this work was made for me by Messrs. E. T. Pearson and Co., Ltd. (Mitcham), to whom I am indebted for the above analysis.
† For 3 weeks and then cod-liver oil 4 cc., cotton-seed oil 10 cc.
‡ For 4 weeks and then 12 g. butter.

The changing of the fats in the above diets needs explanation. The experiment with diet 1 was made first, after which it was decided to use oils instead of butter, because cod-liver oil is regarded as a more constant source of vitamins A and D than is butter, since large quantities of the oil can be obtained at once and the vitamin content of butter varies with the feed of the cow, and also gives definite seasonal variations. When it was discovered that the does produced no litters on the oil diets, butter was substituted because the fertility on diet 1 was satisfactory, hence the change from oils to butter in experiment with diet 3. These changes in the fat ration in the early weeks of the experiments appear to make no difference to the final results, because breeding took place on diets 1 and 3 and not on diets 2 and 4.

Results.

(*a*) *Growth.*

All six diets provided for growth, but the rate differed somewhat as shown in Fig. 1. This point will be referred to later. In each experiment the rats were in excellent condition; they were firm to feel and their coats were thick and sleek.

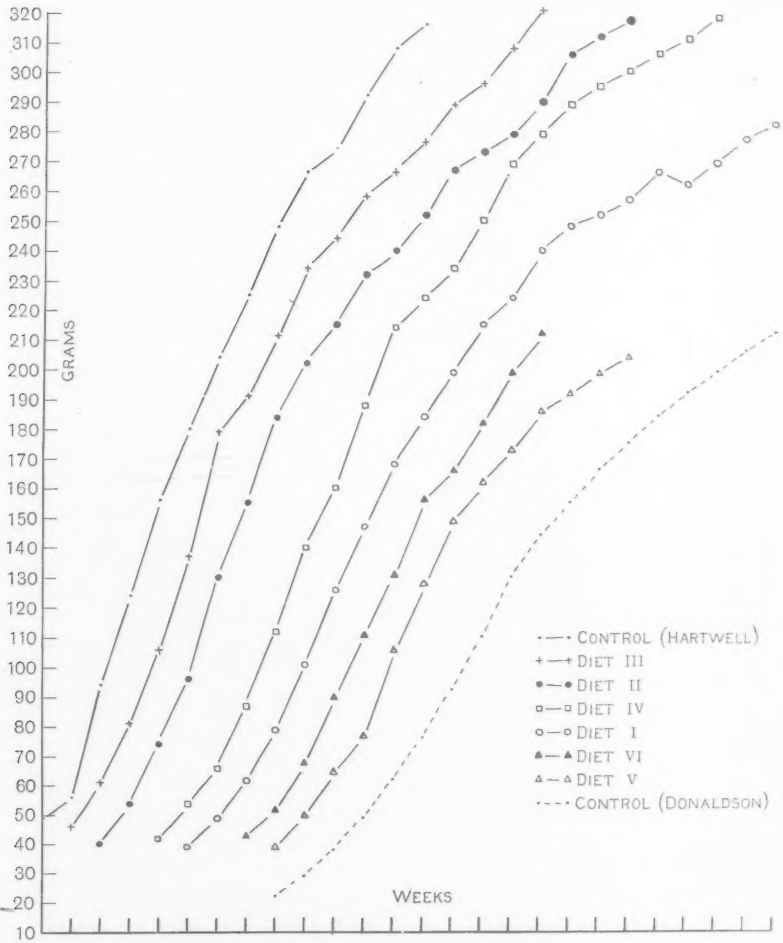

Fig. 1. Growth curves of male rats. Each curve is taken from the average of 6 animals (except with diet IV when only 5 were used).

(*b*) *Reproduction.*

The different experiments gave interesting results as regards reproduction. A summary of the breeding on the 6 diets is given below.

Diet 1. This food was given to two groups of does, one consisting of six animals which received the synthetic diet from 23 days of age (their weights ranging from 35–41 g.) and the other, a group of four does, which when the experiment was started weighed 57, 60, 63 and 68 g. respectively. During gestation, the diet contained 6 g. marmite instead of 2 g.

Group A. Duration of experiment: April 24, 1925—March 1, 1926. One doe died when her first litter was born, the other five animals produced 8, 6, 5, 8 and 9 litters respectively.

Group B. Experiment from April 17, 1925—March 1, 1926. One doe developed a prolapsed uterus at the birth of her second litter, the other three had 8, 6 and 6 litters respectively.

In both groups there was noticeable failure in rearing the young, but Group B gave decidedly better results than Group A. In both groups the litters were of small size and the young of low weight; frequently they were devoured by their mother soon after birth. From Group A 14 young were reared, while from Group B 28 were successfully weaned.

Diet 2. Experiment from June 29, 1925—August 9, 1926. During this time none of the does showed any signs of pregnancy.

Diet 3. This experiment is still in progress; so far each doe has produced 2 litters.

Diet 4. Experiment from August 29, 1925—August 9, 1926. There were five does on this diet and no litters were born.

Diets 5 *and* 6. These experiments were not continued long enough for testing the fertility of the rats.

Discussion.

(*a*) *The composition of the diets.*

(1) *Protein.* It is customary to use caseinogen as the sole source of protein in synthetic diets, probably because there is so little choice of purified proteins. Man does not live on one protein only, and the inefficiency of a synthetic diet may be due to poorness in this respect. In milk, which is regarded as a perfect food for the young animal, the caseinogen is supplemented by lactalbumin. An attempt was made to remedy this possible protein deficiency by supplementing the caseinogen with edestin (diets 5 and 6), but the growth curve showed no indication of improvement, and after 12 weeks the experiments were discontinued. In diet 4 the protein was given in the form of food casein and gluten, thus providing a mixture of proteins. In this experiment the growth was good, though not quite up to that obtained when the rats had a mixed diet, and no better than with other diets containing pure caseinogen only. Diet 3 produced a slightly better growth curve than either of the other

synthetic diets. This food contained more marmite than any of the other diets and it is just possible that the yeast proteins (or protein products) were effective supplements to the caseinogen.

(2) *Vitamin B.* Diet 1 contained only 2 g. marmite per 100 g. solid, which may be regarded as rather little. This amount provided for quite good growth, though the rate was somewhat slower than when more vitamin B was given. Also this lower level of intake was sufficient for reproduction, because the does on diet 1 bred well. This is in agreement with the findings of Guest, Nelson, Parks and Fulmer [1926, 1], who state that the amount of vitamin B required for reproduction is not much greater than that required for normal growth. It is probable that 2 g. marmite is almost enough for maximal growth and 5 g. is excess, because there is only a slight difference between the growth curves obtained with these two diets.

(b) Growth and reproduction.

It is evident from the experiments described in this paper that dietary requirements for growth and reproduction are totally different. The growth on diet 1 was less good than that on diets 2 and 4, yet the does receiving diet 1 produced numerous litters, while those fed on diets 2 and 4 gave birth to no young at all. From an analysis of the composition of the different diets, it appears that the fat is the primary factor in this respect. When butter was given reproduction occurred, but when cod-liver oil and cotton-seed oil constituted the fat ration, no young were born. According to Sure [1924, 1] the absence of vitamin E from the diet would explain these results. Butter is considered rich in this new vitamin, while cod-liver oil is low or lacking in it [Evans and Bishop, 1923]. On the other hand, Sure [1924, 2] obtained fertility in his rats with cotton-seed oil provided it was present to the extent of 5 %, and in the experiments described here the sterile rats were getting 10 % of cotton-seed oil. Mattill and Clayton [1926] also consider that vitamin E is necessary for fertility in both male and female rats, but Anderegg [1924], from similar experiments, concludes that it is unnecessary to assume the existence of a new vitamin for reproduction. Beard [1925] confirms the work of Evans and Bishop that a new factor, vitamin E, is necessary for reproduction.

The amounts of the fat-soluble vitamins in the diets described in this paper vary, and it may be that in diets 2 and 4 there is enough of these factors for growth, but insufficient for reproduction. The amount of cod-liver oil used should provide more vitamin A than is needed for growth, unless the cotton-seed oil possesses some inactivating action, as suggested by Fridericia [1924] for other oils.

Sherman and MacLeod [1925] found that rats on a diet containing relatively little vitamin A grew nearly to adult size, but were shorter lived, and their reproduction was poor compared with those on a similar diet, but containing about seven times as much vitamin A. It is difficult to reconcile the general fitness and good growth of the rats with an actual dietary deficiency, and it

seems probable that a more correct explanation is that offered by Nelson, Jones, Heller, Parks and Fulmer [1926] who suggest that failure in reproduction and lactation is more likely due to inadequate quantitative relations between the known constituents of the diet. It is now recognised that the amount of vitamin B necessary for normal lactation is much greater than that required for normal growth and reproduction [Guest, Nelson, Parks and Fulmer, 1926, 2]. Grant [1924] and Hartwell [1925, 3] find that the lactating rat needs four times as much as the non-lactating rat. It may be that a correspondingly greater quantity of vitamin A is needed for reproduction as compared with growth. The rats fed on diets 2 and 4 produced no litters, although they were in excellent condition. Post-mortem examinations showed that the testes of these animals were of low weight; the uteri also were abnormal. Histological preparations are now being made and will be discussed in a later paper. Experiments are also in progress to determine whether there is a failure in development of the reproductive systems or degeneration of the organs as soon as they mature.

SUMMARY.

1. Descriptions are given of various synthetic diets which produce good but not maximal growth; in one experiment, the growth curve approximates closely but is not quite equal to that obtained from control rats fed on a mixed diet.

2. The dietary requirements of the rat vary considerably at different phases of its existence. Two diets are described which provide for good growth (almost maximal), but no young were born. Another diet which gave less good results for growth was adequate for reproduction.

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CLX. YEAST EXTRACT AS A SUPPLEMENT TO GELATIN.

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GELATIN is recognised as a biologically poor protein, as it contains no tryptophan and only a little tyrosine. Commercial yeast extract may contain protein or protein products, either of which might supplement a poor protein, since yeast proteins have been shown to be of good value. Osborne and Mendel [1919] found that rats grew well on a diet in which yeast furnished the sole source of nitrogen in the food, and Nelson, Heller and Fulmer [1923] have reared third generation animals with yeast as the only source of protein in the diet. Hawk, Smith and Holder [1919] found that bakers' compressed yeast formed a satisfactory article of diet for man. The following experiments were made with a view to finding how far marmite could supplement gelatin.

EXPERIMENTAL.

The diet consisted of:

Sheet gelatin	20 g.	Cod-liver oil	4 cc.
Potato starch	64 g.	Cotton-seed oil	10 cc.
Salt mixture	4 g.	Water	75 cc.

The gelatin was soaked in some of the water for at least half an hour, otherwise, the method used for preparing the diet was that described previously as the "cooked" method [Hartwell, 1925].

Three diets were given:

1. Gelatin diet.
2. Gelatin diet + marmite (5 g. marmite added to the above ration).
3. Gelatin diet + extract from marmite [see Hartwell, 1922]. 5 cc. extract to the above ration (*i.e.* the equivalent of 5 g. marmite).

Each diet was given to 12 young rats, 6 ♂ and 6 ♀; the animals were weighed daily.

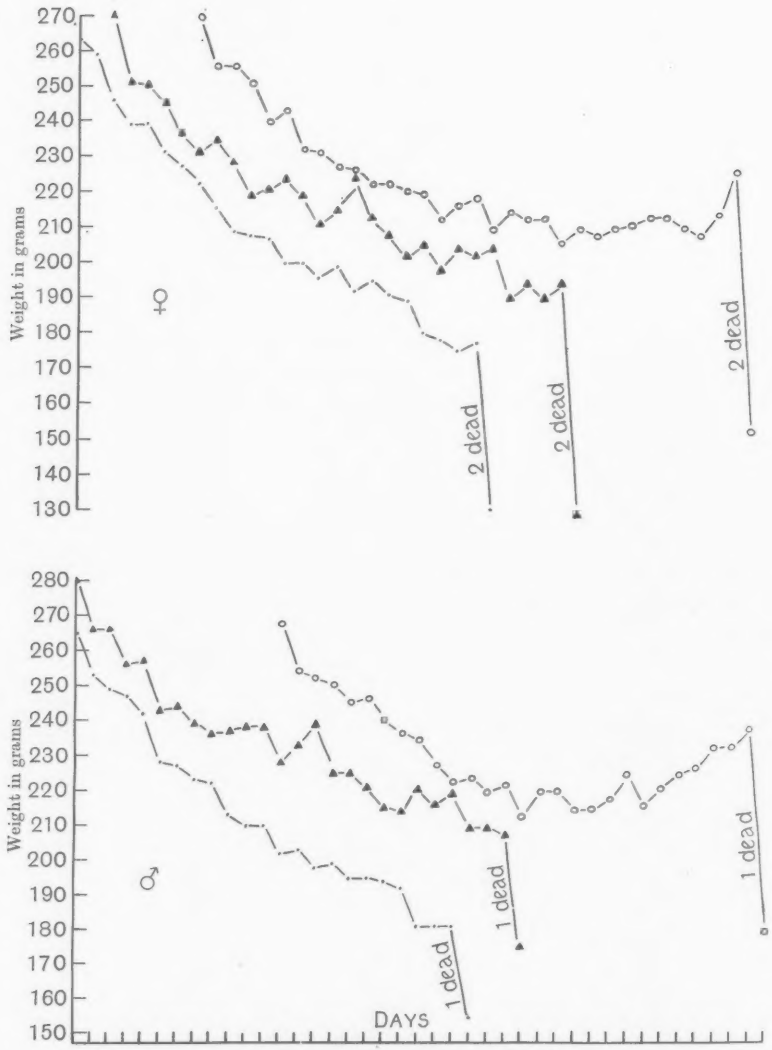

Fig. 1. Each curve represents the combined weights of 6 young rats.

Gelatin diet

Gelatin diet + marmite

Gelatin diet + extract from marmite

Results.

The combined weight curves are shown in Fig. 1. The addition of marmite to the gelatin diet obviously prevented some loss of weight in the young rats, and in some of the animals even led to an increase. This is shown by the following table:

Diet	Total loss of weight g.	Actual gain while on the diet g.
Gelatin	175	15
Gelatin + extract	160	36
Gelatin + marmite	131	49

Comments.

The gelatin used gave a faint Millon's reaction and a negative glyoxylic acid test. A 5 % solution of marmite contains a substance which is precipitated by metaphosphoric acid and also by alcohol; the solution also gives positive reactions with the Millon's and glyoxylic acid tests. From this point of view the yeast extract should supplement the poor protein, and the experiments described above show that the animals lost less weight when marmite was added to the gelatin food. The amount used was the quantity which is generally employed in synthetic diets. The extract made from marmite on the other hand contained only a trace of tryptophan and was of little use in supplementing the poor protein. Since yeast extract can, even to a slight extent, supplement such a poor protein as gelatin, it is obvious that yeast extracts should not be added to a synthetic diet when testing the adequacy of a protein, for a slight deficiency in this factor might well be masked by the extract.

It may be suggested that the animals on the gelatin diet were suffering from lack of vitamin B, but the similarity of the growth curves of those fed on the gelatin diet + extract (which is obviously rich in vitamin B) is evidence against this suggestion. Also it is probable that the gelatin was by no means free from vitamin B. The rats showed none of the nervous symptoms due to lack of vitamin B, and since the "cooked" method of preparing synthetic diets has been adopted, no rats have died on diets free from vitamin B without showing typical nervous symptoms for a day or two before death.

Summary.

Yeast extract supplements gelatin in a synthetic diet.

The expenses of this research were defrayed by a grant from the Medical Research Council.

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CLXI. THE DISTRIBUTION OF XANTHINE OXIDASE I.

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(*Received October 29th, 1926.*)

In a previous paper by Morgan, Stewart and Hopkins [1922] it was shown that "xanthine oxidase" acts as a typical dehydrase in the sense of Wieland. Tissue preparations which contain the enzyme oxidise the bases hypoxanthine and xanthine to uric acid anaerobically in the presence of methylene blue, the dye being reduced to its colourless leuco-compound.

The use of this anaerobic technique, compared with the method of adding known amounts of the bases to a tissue preparation and estimating at the end of the experiment the amount of unchanged xanthine, or hypoxanthine, is so simple that it becomes comparatively easy to test for the presence or absence of the catalyst in the case of any given tissue. As only small amounts of material are required it has the additional advantage that the organs of the smaller animals can be investigated and a large number of organs can be easily compared at the same time.

It was the purpose of the present research to extend the very limited number of animals in which the distribution of the enzyme has been hitherto studied, and its results yield further evidence to show that the xanthine oxidase, like the other enzymes concerned with the breakdown of purines, has a peculiar and limited distribution among the organs of a given species, while the localities in which it is found vary considerably from species to species.

Résumé of the method.

The tissue preparations are conveniently made by Bach's method. The organs from which the preparations are to be made are first freed from obvious fat and connective tissue. The larger organs are then passed through a mincer and sampled. The stomach and intestines are thoroughly washed out with water before using.

The smaller, and from 5 to 10 g. of the larger, organs are ground up with 5 or 10 volumes of 2 % sodium fluoride solution in a mortar, using a little washed sand, and squeezed through strong, fine muslin. It is better to pound the tissue well with the sand before adding a portion of the measured sodium fluoride solution and grinding. After squeezing this through muslin, the débris from the muslin is returned to the mortar and again ground, using the rest of the sodium fluoride solution; after squeezing this through the muslin only connective tissue should remain.

It is important that the preparations should not be filtered through filter paper, for Dixon and Thurlow [1924, 1] have shown that the enzyme is readily absorbed by paper.

The preparations, which are essentially cell suspensions, are transferred to Erlenmeyer flasks, lightly plugged with cotton wool, and allowed to stand with an occasional shake at room temperature, or better at 25°, for 24 to 48 hours, when their original power to reduce methylene blue will be greatly diminished.

From 2 cc. to 5 cc. of the preparations, which should be well shaken and if necessary again squeezed through muslin to obtain a good sample, are then measured into two vacuum tubes similar to those used by Thunberg and described by Hopkins and Dixon [1922]. To one is added a measured quantity of hypoxanthine solution, and to the control the equivalent amount of solvent. To each tube is added the same quantity of methylene blue solution. The tubes are well evacuated, placed in a thermostat with a glass side at 37°, and the reduction time noted.

In the following experiments 0·2 cc. to 0·5 cc. of a freshly prepared $M/100$ solution of hypoxanthine was used, dissolved either in $N/50$ sodium carbonate solution, or in 0·2N NaOH solution and buffered to p_H 7·8 with 0·2M KH_2PO_4. The solution in phosphate buffer was used with tissue preparations which are only slightly buffered and in which, upon adding the $N/50$ sodium carbonate solution, the p_H was above 8·0. Dixon and Thurlow [1924, 2] have shown that the reaction velocity is unaffected by p_H between 5·5 and 9·0.

If it is found that the reducing power of any of the preparations, without added hypoxanthine, is too high to give a blank of 2 to 3 hours using 0·5 cc. and from 3 to 6 hours using 1·0 cc. of $M/1000$ methylene blue solution, they can either be left to stand longer before use, or diluted with an equal volume of 2 % sodium fluoride solution. Otherwise the amount of methylene blue can be increased.

When increasing the strength of the methylene blue the following data should be remembered. 1 cc. of $M/1000$ methylene blue solution is equivalent to 11·2 mm.^{3} of oxygen at N.T.P.; 1 cc. of $M/100$ hypoxanthine solution (0·00136 g.) oxidised to uric acid is equivalent to 224 mm.^{3} of oxygen at N.T.P., which is equivalent to 20 cc. of $M/1000$ methylene blue solution.

The animals were either killed at the slaughter-house and the organs brought fresh to the laboratory, or they were chloroformed in the laboratory and bled from the jugular vein.

It is important that the animals should have no fresh milk for several hours before killing or a positive result may be obtained for the stomach and small intestine.

With the exception of the smaller organs, five volumes of 2 % sodium fluoride were used throughout, and the preparations allowed to stand 24 to 48 hours. In the case of the earthworm and caterpillar, which darken upon standing, 2 cc. of methylene blue were used and the preparations tested after standing for 2 hours without shaking with air.

It would be satisfactory if the evidence could be given *in extenso* but this would call for much space. The full data are therefore presented for three animals only, but when they are recorded in abbreviated form the evidence obtained was, in all cases, equally unequivocal. The result of adding hypoxanthine to a preparation is, in the case of certain organs, to reduce the time necessary for the reduction of a standard quantity of methylene blue so greatly that hours may become minutes. In the case of other organs there is, within the limits of observational error, no effect at all. Only in the rarest of instances is the effect upon the reduction time small enough to be equivocal. The evidence for the presence of the enzyme in any given case is therefore always beyond doubt, and so, with certain qualifications to be discussed later, is the evidence that in another case it is absent. That the actual reduction time in the controls (tissue preparations without added purine base) varies somewhat widely is due, in the first place, to variations in the general reducing powers of different tissues, and, in the second, to variations in the degree to which reducing systems in a given tissue were exhausted before its preparations were employed in an experiment. The latter factor was intentionally varied by varying the time of standing before employment. Examination of the data will show that the definiteness of the results was not affected by any such variation in the controls. All comparisons were of course simultaneous and the only difference between one system and the other was in each case the presence or absence of added hypoxanthine.

Mammals.

Table I. *The Dog.*

R.T. = Time for complete reduction.

A plus sign in the minutes column indicates that the observation ceased before reduction was complete, no difference being then observable between the two tubes compared.

Tissue preparation		R.T. control	R.T. hypoxanthine	Xanthine oxidase
Liver	Dog 1	2 hrs. 40 mins.	2 hrs. 40 mins.	−
	,, 2	4 ,, 0 ,,	4 ,, 7 ,,	−
	,, 3	2 ,, 10 ,,	2 ,, 8 ,,	−
	,, 4	4 ,, +	4 ,, +	−
Kidney	,, 1	2 ,, 40 ,,	2 ,, 45 ,,	−
	,, 2	2 ,, 52 ,,	2 ,, 52 ,,	−
Spleen	,, 1	4 ,, 10 ,,	0 ,, 45 ,,	+
	,, 2	2 ,, 51 ,,	0 ,, 24½ ,,	+
Heart	,, 1	3 ,, 25 ,,	3 ,, 25 ,,	−
	,, 2	2 ,, 43 ,,	2 ,, 46 ,,	−
Muscle	,, 1	3 ,, 2 ,,	3 ,, 8 ,,	−
	,, 2	5 ,, +	5 ,, +	−
Lung	,, 1	6 ,, 7 ,,	0 ,, 33 ,,	+
	,, 2	5 ,, 4 ,,	0 ,, 15 ,,	+
Pancreas	,, 1	5 ,, 10 ,,	5 ,, 16 ,,	−
	,, 2	5 ,, 20 ,,	5 ,, 14 ,,	−
Thymus	,, 1	5 ,, 6 ,,	5 ,, 6 ,,	−
	,, 2	5 ,, 40 ,,	5 ,, 36 ,,	−
Stomach	,, 1	3 ,, 9 ,,	3 ,, 12 ,,	−
	,, 2	6 ,, 14 ,,	6 ,, 23 ,,	−
Small intestine	,, 1	3 ,, 50 ,,	0 ,, 21½ ,,	+
	,, 2	3 ,, 52 ,,	0 ,, 13 ,,	+
Large intestine	,, 1	7 ,, +	7 ,, +	−
	,, 2	3 ,, 35 ,,	3 ,, 35 ,,	−

For comparison of the methylene blue technique with the method of adding the purine bases to a tissue preparation and estimating at the end of the experiment the amount of unchanged xanthine or hypoxanthine and uric acid, I have used the organs of the dog. Using the latter method, xanthine oxidase has been shown by Jones and Austrian [1906] and Schittenhelm [1909] to be present in dog's spleen, intestine and lung, but absent from the liver, kidney, pancreas and muscle. It will be seen from Table I that the methylene blue technique very sharply confirms these authors.

Table II. *The Cat.*

Tissue preparation		R.T. control	R.T. hypoxanthine	Xanthine oxidase
Liver	Cat 1	2 hrs. 10 mins.	0 hrs. 17 mins.	+
	„ 2	1 „ 2 „	0 „ 9 „	+
	„ 3	1 „ 16 „	0 „ 14½ „	+
	„ 4	2 „ +	0 „ 18 „	+
Kidney	„ 1	2 „ 13 „	2 „ 13 „	−
	„ 2	3 „ 5 „	3 „ 9 „	−
	„ 3	3 „ 10 „	3 „ 7 „	−
Spleen	„ 1	9 „ +	9 „ +	−
	„ 2	7 „ +	7 „ +	−
Heart	„ 1	7 „ +	7 „ +	−
	„ 2	3 „ 18 „	3 „ 21 „	−
Muscle	„ 1	3 „ 5 „	3 „ 5 „	−
	„ 2	8 „ +	8 „ +	−
Lung	„ 1	1 „ 42 „	0 „ 12½ „	+
	„ 2	8 „ +	0 „ 50 „	+
	„ 3	5 „ 25 „	0 „ 58 „	+
Pancreas	„ 1	8 „ +	1 „ 22 „	+
	„ 2	8 „ +	1 „ 44 „	+
	„ 3	6 „ 10 „	0 „ 50 „	+
Thymus	„ 1	10 „ +	10 „ +	−
	„ 2	8 „ +	8 „ +	−
Stomach	„ 1	6 „ 40 „	6 „ 40 „	−
	„ 2	7 „ +	7 „ +	−
Small intestine	„ 1	7 „ +	0 „ 33 „	+
	„ 2	8 „ +	0 „ 24 „	+
Large intestine	„ 1	4 „ 17 „	4 „ 10 „	−
	„ 2	7 „ +	7 „ +	−

Table III. *The Sheep.*

Tissue preparation		R.T. control	R.T. hypoxanthine	Xanthine oxidase
Liver	Sheep 1	1 hrs. 50 mins.	0 hrs. 7 mins.	+
	„ 2	1 „ 33 „	0 „ 6 „	+
Kidney	„ 1	2 „ 28 „	2 „ 30 „	−
	„ 2	2 „ 58 „	2 „ 58 „	−
Spleen	„ 1	2 „ 40 „	2 „ 40 „	−
	„ 2	5 „ 5 „	5 „ 17 „	−
Heart	„ 1	3 „ 10 „	3 „ 6 „	−
	„ 2	3 „ 50 „	3 „ 55 „	−
Muscle	„ 1	2 „ 15 „	2 „ 15 „	−
	„ 2	4 „ 39 „	4 „ 35 „	−
Lung	„ 1	3 „ 8 „	3 „ 13 „	−
	„ 2	9 „ +	9 „ +	−
Pancreas	„ 1	2 „ 33 „	2 „ 33 „	−
	„ 2	3 „ +	3 „ +	−
Thymus	„ 1	4 „ 31 „	4 „ 31 „	−
	„ 2	3 „ 53 „	3 „ 49 „	−
Stomach	„ 1	10 „ +	10 „ +	−
	„ 2	6 „ +	6 „ +	−
Small intestine	„ 1	10 „ +	10 „ +	−
	„ 2	8 „ +	8 „ +	−
Large intestine	„ 1	5 „ 46 „	5 „ 50 „	−
	„ 2	7 „ +	7 „ +	−

The figures in the preceding experimental data have been selected from a number of duplicates. A difference of a few minutes in the reduction times of a control tube and of one to which hypoxanthine has been added, in the case of a preparation which takes several hours to reduce, can be disregarded as falling within the limits of experimental error. In the following shortened presentation of the data obtained from other animals, the figure after the name of an organ indicates the number of individual animals studied in each case.

The Goat.

Liver (2) +. Kidney (2) −. Spleen (2) −. Heart (2) −. Muscle (2) −. Lung (2) −. Pancreas (2) −. Thymus (2) −. Stomach (2) −. Small intestine (2) −. Large intestine (2) −.

The Rabbit.

Liver (2) +*. Kidney (2) −. Spleen (2) −. Heart (2) −. Muscle (2) −. Lung (2) −. Pancreas (2) −. Thymus (2) −. Stomach (2) −. Small intestine (2) +. Large intestine (2) −.

* Xanthine oxidase has been shown to be present in rabbit's liver by Jones and Austrian [1906]. I have confirmed the work of these authors.

The Hedgehog.

Liver (3) −. Kidney (2) −. Spleen (2) −. Heart (2) −. Muscle (2) −. Lung (2) −. Pancreas (2) −. Thymus (2) −. Stomach (2) −. Small intestine (2) +. Large intestine (2) −. Mammary gland (active) (2) +.

BIRDS.

The Pigeon.

Liver (3) −. Kidney (2) +. Heart (2) −. Muscle (2) −. Lung (2) −. Pancreas (3) +. Gizzard (2) −. Small intestine (2) −. Large intestine (2) −.

The Fowl.

Liver (5) +. Kidney (2) +. Spleen (2) −. Heart (2) −. Muscle (2) −. Lung (2) −. Pancreas (5) −. Gizzard (2) −. Small intestine (2) −. Large intestine (2) −.

The remarkable contrast in respect of their content of the enzyme displayed by the liver of closely related species is so noteworthy as to justify the experimental data being given in full (Table IV).

Table IV.

Liver preparation	R.T. control	R.T. hypoxanthine	Xanthine oxidase
Dog 1	2 hrs. 40 mins.	2 hrs. 40 mins.	−
„ 2	4 „ 0 „	4 „ 7 „	−
„ 3	2 „ 10 „	2 „ 8 „	−
„ 4	4 „ +	4 „ +	−
„ 5	2 „ 6 „	2 „ 6 „	−
„ 6	2 „ 18 „	2 „ 22 „	−
Cat 1	2 „ 10 „	0 „ 17 „	+
„ 2	1 „ 2 „	0 „ 9 „	+
„ 3	1 „ 16 „	0 „ 14½ „	+
„ 4	2 „ +	0 „ 18 „	+
Fowl 1	1 „ 15 „	0 „ 22 „	+
„ 2	2 „ 3 „	0 „ 29 „	+
„ 3	1 „ 18 „	0 „ 2½ „	+
„ 4	2 „ 6 „	0 „ 4 „	+
„ 5	1 „ 25 „	0 „ 8 „	+
Pigeon 1	4 „ 27 „	4 „ 27 „	−
„ 2	3 „ 9 „	3 „ 6 „	−
„ 3	3 „ 7 „	3 „ 13 „	−

Invertebrates.

The Lobster.

Liver (2) −. Green gland (3) −. Heart (2) −. Muscle (2) −. Gills (2) −. Stomach (2) −. Testes and seminal vesicles (2) −. Blood (2) −.

The Earthworm.

Whole worms (4) −. Pharynx −. Crop and gizzard −. Intestine (upper) −. Intestine (lower) −. Nephridia −. Sections containing hearts −. Muscle −. Seminal vesicles −*.

* Combined organs from a large number of earthworms.

I also found the enzyme to be absent from the oyster, and present in the caterpillar and pupae of *Pieris brassicae*. It is also present in rat carcinoma.

In Table V most of the available data obtained by others, as well as by myself, are collected together, so that the varying distribution of the enzyme can be clearly seen. The references to the previous literature of the data given are contained in Abderhalden [1911], Oppenheimer [1911], Wells [1910], Caldwell and Wells [1914] and Morgan, Stewart and Hopkins [1922]. The data furnished by other observers are marked by an asterisk.

Table V.

	Man	Monkey	Ox	Horse	Sheep	Goat	Pig	Dog	Cat	Rabbit	Rat	Opossum	Hedgehog	Pigeon	Fowl
Liver	+*	+*	+*	?	+	+	+*	−*	+	+*	+*	+*	−	−	+
Kidney	−*	−*	+*	?	−	−	−*	−*	−	−	+*	+*	−	+	+
Spleen	−*	−*	+*	+*	−	−	−*	+*	−	−	+*	−*	−	?	−
Heart	?	−*	?	?	−	−	?	−	−	−	?	?	−	−	−
Muscle	−*	−*	−	?	−	−	−*	−*	−	−	−*	−*	−	−	−
Lung	−*	−*	+*	?	−	−	−*	+*	+	−	+*	−*	−	−	−
Pancreas	−*	−*	−	?	−	−	−*	−*	+	−	?	?	−	+	−
Thymus	?	?	−*	?	−	−	?	−	−	−	?	?	−	?	?
Stomach	?	−*	?	?	−	−	−*	−	−	−	?	?	−	−	−
Small intestine	+?*	−*	+*	?	−	−	−*	+*	+	+	?	−*	+	−	−
Large intestine	?	−*	?	?	−	−	?	−	−	−	?	?	−	−	−
Mammary gland (active)	?	?	?	?	?	?	?	?	?	?	?	?	+	.	.

Discussion.

As was earlier indicated, and as will be seen on examination of the results given in the tables, wherever the presence of the enzyme in a tissue has been claimed in this research, the evidence yielded by the use of the anaerobic technique has been unequivocal. Whenever the absence of the enzyme has been claimed the effect of such addition has been, in practically every case, completely negative.

The negative results, however, call perhaps for some comment. It is well known, of course, that throughout a certain range in the relative concentration of any enzyme and its substrate an increase in the latter may fail to increase the velocity of reaction, the enzyme surface being saturated from the beginning. In a given tissue extract or cell suspension, such as those used in this research,

the breakdown of nuclein materials may be in progress, and if the production of xanthine and hypoxanthine from their precursors is faster than their destruction, they will accumulate while the preparation is allowed to stand. The oxidase, if present in relatively low concentration, might then be saturated with its substrate when the experiment is begun, so that no decrease in reduction time would follow upon the addition of purine base. When, however, tissue preparations are allowed to stand long enough before use to render their reducing power for methylene blue so small as to be negligible, the addition of base is still without effect in cases where the absence of the enzyme has been claimed, but always enormously decreases the reduction time in the case of tissues which undoubtedly contain the enzyme. The latter show that the catalyst is not readily destroyed under the conditions of experiment. It would seem, therefore, that the considerations put forward in the last paragraph have no important bearing on the results. When, with the technique employed, the addition of purine base has no observable effect upon reduction time, the enzyme cannot be present in a concentration which would be physiologically significant.

The suggestion may arise that, when a tissue fails to oxidise xanthine or hypoxanthine, this is due not to the absence of the active catalyst, but to the presence of an inhibitory factor. The results of such experiments as the following speak against such a view. The spleen of the ox is rich in the enzyme, whereas, if the general evidence be accepted, that of the pig contains none. A preparation was made of each, and both preparations were allowed to stand until their reducing power for methylene blue was very small, and their uptake of oxygen in Barcroft's apparatus negligible. The ox spleen preparation was then mixed (*a*) with an equal volume of the pig spleen preparation, and (*b*) with an equal volume of sodium fluoride solution. All conditions being the same each mixture exhibited the same oxidative power, anaerobically and aerobically. Each by itself showed a reduction time of about 4 hours (concentrations being as in the majority of the experiments reported). In each case the addition of the standard amount of hypoxanthine shortened the reduction time to 12 minutes. In the Barcroft apparatus neither mixture showed any significant uptake of oxygen. When to 2 cc. of each, 1 cc. of $M/100$ hypoxanthine solution was added, the oxygen uptake was identical (286 mm.^{3} in 11 hours) and the velocity curves were nearly superposable. The oxidation was clearly due to the ox spleen alone, whilst the pig spleen was without effect. Such results may not wholly prove the absence of an inhibitory factor from the latter; but they make against a hypothesis which is in itself not very likely.

A survey of the results given in Table V leads to the following conclusions.

The enzyme is present in the livers of ten out of the twelve mammals examined. It is however absent from that organ in the case of the dog and the hedgehog. The difference in this respect between the carnivores, the dog and the cat respectively, is so definite and noteworthy that full details are

given in Table V. Its absence from the liver of the dog had been shown previously by Jones and Austrian [1906] and my own observations upon six individuals fully confirm the circumstance. That it is present in cat's liver is equally sure.

It is absent from the kidney of all the mammals examined[1] save in the case of the opossum. In this its appearance would seem to be variable [Caldwell and Wells, 1914], a circumstance not without interest in the case of a marsupial, since its presence in the kidney of birds—to judge from the two species examined—seems definite.

In the case of mammalian organs other than the liver and kidney its distribution is highly variable from species to species. In man and in monkeys it may be confined to the liver though all the organs have not been examined. In three ungulates (sheep, goat and pig) it appears to be wholly confined to that organ. In the ox on the other hand it is certainly also present in the spleen, lung, and small intestine, and the cat shows a similar distribution save for its absence from the spleen and presence in the pancreas. The hedgehog offers a case in which under ordinary circumstances it would seem to be only in the small intestine. I found however that the enzyme is present in highly efficient concentration in the active mammary gland of the last-named animal, and also in that of the guinea-pig. Since the presence of the enzyme is characteristic of milk a further investigation of the mammary gland, passive and active, would be of interest. Taking the rat as an example of a small animal with a high basal metabolism it is to be noted that the enzyme is here probably widely distributed; the negative results of Rohde and Jones [1909] have been explained by Dixon and Thurlow [1924, 1]. It is certainly present in the liver, kidney and spleen, and also in the lung; other organs in this animal have unfortunately not been examined. Save the fact that it is generally present in the liver and absent from the kidney of mammals, and to the former at least of these statements there is in the dog a noteworthy exception, no general statement is possible.

No genetic relationships in the distribution are obvious, though, to decide concerning the possibility of these, many more representative species would have to be studied.

It would not be surprising to find where a factor of purine metabolism is concerned that birds differ from mammals. To judge from the two cases I have studied such a difference is found in the unquestionable presence of the xanthine oxidase in the avian kidney. Here again, however, we find a remarkable difference between species; the enzyme is certainly present in the liver of the fowl, and with equal certainty absent from that of the pigeon.

It would be interesting to know whether in the metabolism of species with

[1] The kidney of the ox is exceptional in not yielding the clear-cut results (positive or negative) given by other tissues. My observations suggest that it contains the enzyme in very low concentration. Schittenhelm [1905] has stated that it is present, but if his figures are consulted they will be found to show at most a doubtful activity.

a limited distribution of the enzyme there is a greater escape of xanthine and hypoxanthine than in others which contain it more widely distributed. The only data available on this point are contained in the comparative studies of Hunter and Givens [1914] and of Hunter, Givens and Guion [1914]. Even with these, however, it is impossible to generalise. It may be noted, however, that in the case of the three ungulates, sheep, goat and pig (the pig was studied by Schittenhelm [1910]), the excretion of purine bases is relatively high, and in these animals the oxidase is present in the liver only. In the ox, on the other hand, the excretion of bases is low and the oxidase is widely distributed among its organs.

The identity of xanthine oxidase with the Schardinger enzyme.

Dixon and Thurlow [1924, 2] working with enzyme preparations from milk have put forward certain evidence, which they, however, do not feel is sufficient to justify a positive statement, to suggest that the xanthine oxidase is identical with the Schardinger enzyme.

Although a large number of data obtained by myself seem to lend support to this view they cannot perhaps, be put forward as valid evidence for assuming the identity of the two enzymes. Yet, it is very significant that in all the tissues which I have investigated, where the presence of xanthine oxidase was demonstrated, replacement of the purine base by acetaldehyde in neutral solution, always induced a fairly rapid reduction of the methylene blue. Where, on the other hand, the xanthine oxidase was proved absent, the addition of aldehyde was in many cases without effect. It should be noted, however, that, in some of the tissues which do not contain xanthine oxidase a definite decrease in the reduction time may follow upon the addition of acetaldehyde, when the control is from 6 to 10 hours; this is most marked in sheep's lung. This reduction must be further investigated before any conclusions can be drawn, but, as Dixon [1926] points out, "it is quite certain that there are other enzymes which will oxidise aldehydes, but which are altogether without action upon hypoxanthine."

SUMMARY.

1. The organs of the following mammals, the sheep, goat, rabbit, cat and hedgehog, and those of the domestic pigeon and fowl, have been investigated for the presence of xanthine oxidase. The tissues of the following invertebrates were also studied, the lobster, oyster, the caterpillar and pupae of *Pieris brassicae*.

2. Evidence regarding the identity of xanthine oxidase with the Schardinger enzyme is put forward.

I am greatly indebted to Sir F. G. Hopkins for much helpful advice and for allowing me to undertake this work with the aid of a grant allotted to him by the Medical Research Council.

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CLXII. EFFECTS OF VARIOUS AGENTS ON COLOUR TESTS FOR VITAMIN A.

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In a preliminary communication to the Biochemical Society [Moore and Willimott, 1926], as a result of further experience of Fearon's "pyrogallol" test, experimental evidence was brought forward to show that this is not specific for vitamin A. A more complete account of the grounds for this conclusion is now given.

Applied qualitatively, as found by Fearon [1925], and confirmed by Willimott and Moore [1926], the "pyrogallol" test appeared satisfactory. It was given by all cod-liver oils tested, by butter fat, beef suet, and by egg-yolk fat; it was not given by olive oil, linseed oil, palm kernel oil or by ostelin. The ratio obtained between a potent cod-liver oil and butter fat was reasonable (about 40), and varying the concentration of the oil caused corresponding alterations in the intensity of the colour produced. When, however, the "pyrogallol" test was applied to a series of cod-liver oils, two disconcerting facts emerged. First, the grading of the oils by the "pyrogallol" test did not agree with their reputed potencies. Secondly, oils when tested by our technique and by that of Carr and Price [1926] gave contradictory results. For example, on comparing a Newfoundland and a Norwegian oil, both of 1926 season, the "pyrogallol" test showed the Norwegian to be definitely more potent than the Newfoundland, whereas the antimony trichloride test of Carr and Price indicated that the Newfoundland oil was much more potent than the Norwegian. Rosenheim and Drummond [1925] have produced strong presumptive evidence for the specificity of the arsenic trichloride test. These facts which we had ascertained threw doubt on the belief that the Fearon test was essentially a modification of the arsenic trichloride and related tests. We had already shown that it was quite possible to carry out the tests with the reagents interchanged, using arsenic trichloride suitably diluted with toluene to obtain a pink colour, and using melted trichloroacetic acid to obtain a transient blue. Further experiments have shown the two tests to be quite distinct.

Experimental.

Treatment with sulphuric acid.

A sample of Newfoundland cod-liver oil was taken which had been found by previous feeding experiment to be a potent source of vitamin A. About 25 g. were put in a beaker, and concentrated sulphuric acid was gradually stirred in until no further purple colour developed, about 20 cc. being used. No precipitate was formed. An equal volume of water was added gradually, and after 10 minutes the mixture was transferred to a separating funnel, and the brown acid layer run off. The upper layer was washed many times with distilled water until freedom from acid was shown by the p_H of the washings being the same as that of the original oil (about 5·2). It was then extracted with ether, dried with anhydrous calcium chloride, and the ether distilled off under reduced pressure. The residue consisted of a clear oil only a trifle yellower than before treatment. This was tested with concentrated sulphuric acid, phosphorus pentoxide, arsenic trichloride, antimony trichloride in chloroform, and trichloroacetic acid with pyrogallol and with resorcinol. Negative results were obtained with all except Fearon's reagents, which gave as intense a carmine as before treatment. The Fearon's reagent was a 10 % solution in toluene of a sample of trichloroacetic acid of A.R. standard supplied by the British Drug Houses. This quite unexpected result was then further investigated with a view to confirmation.

It was found that a potent Norwegian cod-liver oil, of 1926 season, on treatment in the same manner with concentrated sulphuric acid gave the same results as had been obtained with the Newfoundland oil, so that the cause could not be attributed to the presence of some constituent peculiar to Newfoundland oil. Using fresh reagents, other samples of different origin again confirmed our findings. There still remained the possibility that the colours given by Fearon's test on cod-liver oils treated with concentrated sulphuric acid were due to sulphonated derivatives. To test this, olive oil was treated in exactly the same manner. No colour was obtained with Fearon's reagent either before or after treatment. The pink colour obtained with Fearon's reagent could not therefore be attributed to sulphonation.

The "sulphonated" oil was next saponified by heating for an hour with 10 % alcoholic potash on a water-bath, and the unsaponifiable matter was extracted with ether and evaporated. The residue gave negative results with Fearon's as well as with all other tests. The soaps obtained from the "sulphonated" oil were next tested, and gave negative results with all except Fearon's, which was faintly positive. Another experiment was performed in which the treated oil was thoroughly shaken with fuller's earth at 110°, but after this treatment a positive Fearon test was still obtained.

From these results a sharp differentiation was obtained. The "sulphonated" oil, according to Fearon's test, was still rich in vitamin A; according to all the other tests, it was devoid of that vitamin.

The "sulphonated" oil was now fed to two albino rats whose growth had been stopped by deficiency of vitamin A, vitamin D having been supplied by direct irradiation. 40 mg. daily doses were given. No growth response was shown, and the animals both developed severe xerophthalmia. Autopsies confirmed vitamin A deficiency. The same oil before treatment had promoted normal growth, and ensured complete protection from eye disease in doses of 16 mg.

Adsorption with phosphorus pentoxide.

Fearon found that when phosphorus pentoxide is mixed with cod-liver oil the sterols are removed by adsorption, and neither they nor the residual oil contain vitamin A, as shown both by feeding experiments and by colour tests. We therefore made use of this destructive action of phosphorus pentoxide on vitamin A in our further investigation of the colour tests.

20 g. of a potent Norwegian cod-liver oil were put in a glass mortar, and powdered phosphorus pentoxide was gradually ground into it. A deep purple colour slowly appeared after each addition of pentoxide, reaching its maximum in 7–10 minutes, and changing to brown on standing, a precipitate being thrown down. When about 10 g. had been added, the supernatant clear oil was tested. With concentrated sulphuric acid negative results were obtained; with phosphorus pentoxide, arsenic trichloride and antimony trichloride faintly positive; with trichloroacetic acid solution to which either resorcinol or pyrogallol had been added, strongly positive. As it appeared from these results that the vitamin had not been completely destroyed, the oil was treated with a further 10 g. of pentoxide, forming a thick paste. This was centrifuged and the supernatant clear oil tested. Negative results were obtained with concentrated sulphuric acid and phosphorus pentoxide; very faintly positive with arsenic trichloride and antimony trichloride; positive with Fearon's reagents.

The results of these experiments, which are summarised in Table I, prove the non-specificity of Fearon's "pyrogallol" test, and in addition show that the arsenic trichloride test of Rosenheim and Drummond and the antimony trichloride test of Carr and Price are more sensitive than the sulphuric acid and phosphorus pentoxide tests. We now sought for a method of vitamin destruction which could be more accurately controlled, and thus permit quantitative applications. Our first attempt was to destroy the vitamin by aeration at 100°. Unfortunately, we found that this produced a darkening in colour which prevented accurate measurement of the intensity given by any of the reactions. Another attempt in which cod-liver oil was heated in a thin film in the presence of air for many hours also proved unsuccessful.

Action of ultra-violet light.

Peacock [1926] has recently published results of an investigation in which cod-liver oil was exposed to the action of ultra-violet light, causing the destruction of vitamin A, as shown both by a negative arsenic trichloride test and by failure to protect from xerophthalmia or to produce growth in rats on a diet otherwise deficient in vitamin A. No details were given, however, as to the time of exposure or distance from the mercury vapour lamp. This method of destroying vitamin A seemed likely to obviate the difficulty of discoloration and, moreover, to permit strict quantitative control.

Table I. *Colour tests on cod-liver oil before and after treatment with agents which destroy vitamin A.*

Oil	Conc. H_2SO_4	P_2O_5	$AsCl_3$	$SbCl_3$	CCl_3COOH + resorcinol	CCl_3COOH + pyrogallol
Untreated	++	++	++	++	++	++
"Sulphonated"—						
(*a*) Ether extract ...	−	−	−	−	+	+
(*b*) Ether extract (after shaking with fuller's earth)	−	−	−	−	+	+
(*c*) Ether extract (saponifiable fraction) ...	−	−	−	−	±	±
(*d*) Ether extract (unsaponifiable fraction)	−	−	−	−	−	−
"Depigmented" (with phosphorus pentoxide)—						
(*a*) Partially	−	±	+	+	++	++
(*b*) Completely	−	−	±	±	+	+

About 50 cc. of a potent Norwegian cod-liver oil were placed in a porcelain evaporating basin of such a size that the oil was not more than 1 cm. deep. It was then exposed at a distance of 7·5 cm. to the radiations from a Hewittic mercury vapour lamp known to be yielding large amounts of rays down to 200$\mu\mu$. The oil was frequently stirred with a thermometer. The temperature rose gradually to about 70°, at which it remained steady, and the natural odour of the oil was noticeably increased. No difference was observed in the colour. At intervals of 15 minutes 2 to 3 cc. of the oil were run off into dry bottles of non-actinic glass, which were kept away from the light and well stoppered. These samples of oil which had been irradiated for different periods of time under the same conditions were then tested for vitamin A with the different colour reactions. The results are recorded in Table II. It will be seen that concentrated sulphuric acid and phosphorus pentoxide both ceased to give positive tests after three-quarters of an hour's irradiation; arsenic trichloride and antimony trichloride did not become negative until after 1½ hours; while Fearon's reaction persisted until the end of the experiment, when over 3½ hours' exposure had been given. Peacock found that cod-liver oil when first exposed to ultra-violet light exhibits a fluorescence, which disappears gradually as the vitamin is destroyed. We have been unable to confirm this observation.

Table II. *Colour tests on cod-liver oil which has been exposed to ultra-violet light for different periods.*

Time exposed (minutes)	Temp. ° C.	Conc. H_2SO_4	P_2O_5	$AsCl_3$	$SbCl_3$	CCl_3COOH + resorcinol	CCl_3COOH + pyrogallol
15	40	+ +	+ +	+ +	+ +	+ +	+ +
30	60	+	+	+ +	+ +	+ +	+ +
45	68	±	±	+ +	+ +	+ +	+ +
60	70	–	–	+	+	+ +	+ +
75	70	–	–	+	+	+ +	+ +
90	70	–	–	±	±	+ +	+ +
105	70	–	–	–	–	+ +	+ +
120	70	–	–	–	–	+ +	+ +
135	71	–	–	–	–	+ +	+ +
150	71	–	–	–	–	+ +	+ +
165	71	–	–	–	–	+ +	+ +
180	70	–	–	–	–	+ +	+ +
210	71	–	–	–	–	+ +	+ +

The experiment was repeated under the same conditions on another sample of the same oil, but withdrawing a few cc. every 5 minutes for the first hour, then every $\frac{1}{4}$ hour until negative arsenic and antimony trichloride tests were obtained. Exposure was continued until the end of $5\frac{1}{2}$ hours, when a positive Fearon test was still obtained.

The next day readings were taken in a Lovibond tintometer of the colour intensities produced on these irradiated oils by the arsenic trichloride and the antimony trichloride reagents. In the case of the first, 0·1 cc. of oil was added by means of a calibrated pipette to 2 cc. of pure arsenic trichloride in a dry test-tube, rapidly mixed by twirling the tube, transferred to a $\frac{1}{4}$-inch cell already in position in the tintometer, and a reading taken as quickly as possible. On account of the transient nature of the colours produced, great difficulties were experienced in obtaining accurate readings, and readings were only approximate below the point where a third of the vitamin had been destroyed. On repeating with pure arsenic trichloride of another make the same results were obtained.

In the case of antimony trichloride, the directions of Carr and Price [1926] were followed, with certain modifications. As reagent we used a saturated solution of antimony trichloride in anhydrous chloroform, approximately 30 % (weight in volume). This was prepared by shaking excess of pure antimony trichloride, previously washed with chloroform, in a stoppered flask at intervals for 12 hours with anhydrous chloroform, and then filtering into a dry stoppered bottle of non-actinic glass. Although Carr and Price state that the solvent in their reagent does not need to be perfectly dry, our experience has been that when it is prepared with the ordinary B.P. chloroform according to their directions, this reagent tends to become cloudy on standing, thus preventing a clear colour being obtained for reading in the tintometer. We therefore dried our chloroform by shaking in a bottle of non-actinic glass for several days with anhydrous sodium sulphate, until it no longer gave a blue colour to anhydrous copper sulphate shaken with it, and a solution prepared from this anhydrous chloroform has given excellent results. A solution of the oil to be

tested, 20 % by volume, was made with this chloroform, and 0·2 cc. of this, equivalent to 0·04 cc. of the oil, was added to 2 cc. of the antimony trichloride reagent. The two were rapidly mixed by twirling, transferred to a ½-inch cell in the tintometer, and a reading taken immediately. The colours obtained, although still transient, lasted distinctly longer than those given by arsenic trichloride, and by taking readings within 15 to 20 seconds of mixing, it was possible to obtain results varying not more than 10 % from one another down to the point where over half the vitamin had been destroyed. Beyond this point the colours, though quite definite, vanished within a few seconds, so that an accurate measurement of their intensity could not be made. It was found that arsenic trichloride and antimony trichioride, when used in these concentrations, gave on the same oil practically the same tintometer readings. As it has been suggested by Rosenheim and Drummond [1925] and Takahashi *et al.* [1925] that the blue colour is indicative of vitamin content, we have used our readings of Lovibond blue units obtained with the arsenic and antimony trichloride reagents to construct the curve in Fig. 1, representing the effect of ultra-violet light on vitamin A in cod-liver oil.

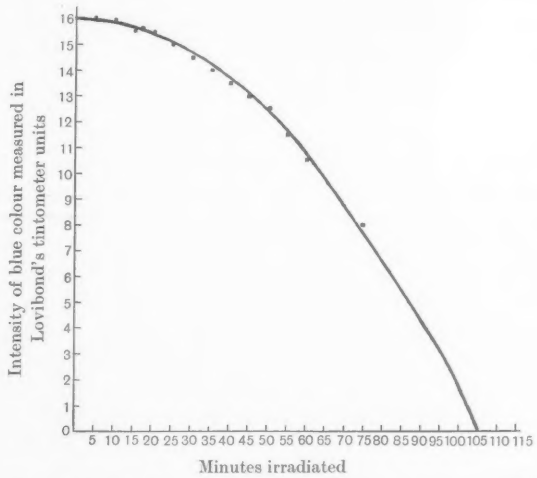

Fig. 1. Effect of ultra-violet light on cod-liver oil, as measured by the arsenic trichloride and antimony trichloride reagents.

We have recently found that when this quantitative method was repeated on oils which had been kept in the dark for several weeks after irradiation, a drop in the reading was obtained, in one instance from 16 to 11 Lovibond units in 14 days. This would seem to indicate that exposure of the oil to ultra-violet light initiates destructive processes which continue for some time after the exposure has been terminated.

SUMMARY.

Cod-liver oil was exposed to the action of the following vitamin A-destroying agents: concentrated sulphuric acid, phosphorus pentoxide and ultra-violet light. The following results were obtained.

1. Fearon's "pyrogallol" test is non-specific. (This conclusion agrees with the findings of Rosenheim and Webster [1926], working on different lines.)

2. Concentrated sulphuric acid and phosphorus pentoxide are less sensitive tests for vitamin A than arsenic trichloride or antimony trichloride.

3. Using the last two tests as criteria of vitamin A content, parallel results were obtained when following the course of destruction of the vitamin by ultra-violet light. Antimony trichloride, however, seems more suitable for use in quantitative methods, since it gives colours which persist longer.

4. In view of the transient nature of the colours obtained with both these reagents, it is suggested that readings be taken not more than 30 seconds after mixing.

We are indebted to Sir F. G. Hopkins for his interest and support, and to Professor E. C. C. Baly for kindly affording us laboratory facilities.

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CLXIII. VITAMINS AND OTHER CONSTITUENTS OF GRAPE-FRUIT RIND.

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A review of the literature reveals the fact that the grape-fruit (*Citrus decumana*) has been little studied either botanically or as regards its vitamin or other constituents. Zoller [1918], whose work on grape-fruit constituents is the most comprehensive we have been able to discover, reports considerable variations in the constituents and therapeutic activity of the different kinds of grape-fruit which are obtainable. He classifies these fruits into two main divisions. One, the larger, very bitter, non-edible kind is frequently termed the shaddock [Hume, 1901]. The other, smaller, less bitter, edible variety is the fruit usually met with in commerce, and was the one we employed in this investigation.

The bitter principle found in grape-fruit is the glucoside naringin. This occurs in various parts of the plant, but its concentration appears to be highest in the peel. It would appear from the work of de Vry [1866] and Pfeffer [1874] that naringin is specific to the grape-fruit. Moreover, the closely related glucoside hesperidin, characteristic of the orange but which has been found in a large number of other plants [Wander, 1925], is not found in the grape-fruit [Pfeffer, 1874; Will, 1885, 1887; Van Rijn, 1900]. We find that naringin can be extracted from the peel by cold alcohol, even when diluted with water, by boiling water or by dilute alkali, but is precipitated by acid and is hydrolysed by prolonged heating above 80°.

It has been shown by a number of investigators [Dominguez, 1918; Lopez, 1920; Hawkins, 1921] that in grape-fruit during the process of maturing there is some relationship between the acidity, sugar and glucosidal content. For this reason, we thought it of interest to obtain data in regard to these points on the material whose vitamin content was being examined.

Preparation of experimental material.

Fresh ripe West Indian grape-fruit, weighing about 350 g., were washed with cold water, and dried. The flavedo was removed with a sharp knife, taking care to include as little as possible of the albedo. The moist flavedo was transferred at once to a well-stoppered bottle kept in the refrigerator, from which the daily doses were weighed out.

The amount of flavedo obtained from each fruit was from 15 to 20 g., or 4 to 5 % of the total weight. The moisture content, ascertained by drying to constant weight at 40°, varied from 70 to 75 %. The p_H of both flavedo and albedo, determined electrometrically, using a quinhydrone electrode, and colorimetrically, as previously described [Willimott and Wokes, 1926], ranged from 4·3 to 4·6, and of the juice from 5·1 to 5·2.

In preparing the alcoholic extract, the fresh flavedo was chopped into small pieces, weighed immediately into a well-stoppered bottle of non-actinic glass, and covered with 90 % alcohol at the rate of 1 cc. to each gram of rind. This was stored in the refrigerator, away from direct light, and shaken at frequent intervals for 3 weeks. It was then filtered, giving a clear golden liquid with a characteristic smell and a markedly bitter taste, due to the essential oil and naringin present. Tests applied to this alcoholic extract showed that the enzyme peroxidase, which we have shown to be present in considerable amounts in grape-fruit rind, had not been extracted by the solvent. Analysis gave the following results:

p_H	4·4
Specific gravity at 15°	0·9497
Total solids	5·74 %
Reducing sugar	2·8 %
Total sugar (after hydrolysis at 40°)	3·6 %

To obtain the oil for feeding to the experimental animals, the fresh rind was air dried for 1 hour at 40°, then put in a stoppered vessel and covered with acetone. This was set aside in the dark and shaken at frequent intervals for a week. It was then filtered, and the residue extracted for another week with dried ether. The mixed acetone and ether extracts were evaporated *in vacuo*, giving a reddish brown oil with an intensely bitter taste. This was kept in a stoppered bottle in the refrigerator. In addition to any vitamins which might be present, it consisted largely of the terpene *d*-limonene, with smaller amounts of citral, *α*-pinene, geraniol and linalool, and traces of various esters.

Feeding experiments.

Vitamin A.

Morgan appears to be the only worker who has published a detailed study of the vitamin A content of grape-fruit. In testing the peel [1923], she used a sample prepared by Mr C. P. Wilson at the Research Laboratory of the California Fruit Growers' Exchange. This had been dried at 65 to 70°, and averaged 34 % of the fresh peel, and 8 to 10 % of the whole fresh fruit. In testing it for vitamin A, the curative method was employed. The rats accepted grape-fruit peel less readily than orange or lemon peel, and although 1 g. doses were administered, the whole of it was not eaten. Her finding was that dried outer grape-fruit peel is ineffective as a source of vitamin A. In a later paper

[Morgan and Chaney, 1924], the rind oil was also tested, and a dose as large as 1 g. was found to contain no appreciable amount of vitamin A. In these experiments the supply of vitamin D does not appear to have been controlled. Moreover, Morgan's results are not in accord with the preliminary findings of Cooper [1921], who states, without giving experimental details, that grape-fruit peel contains fat-soluble vitamin. It seemed to us desirable, therefore, in continuing our studies on the *Citrus* fruits, to investigate the vitamin A content of grape-fruit flavedo, taking the precaution emphasised by Drummond, Coward and Handy [1925] that an adequate amount of vitamin D was supplied.

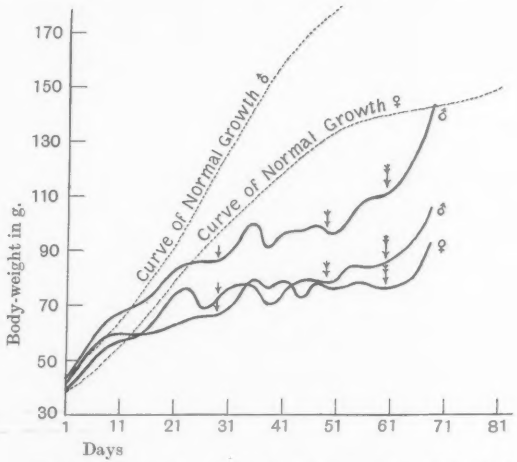

Fig. 1. Vitamin A in grape-fruit flavedo and its 90 % alcoholic extract.
↓ Grape-fruit flavedo, 0·5 g. per head per day.
↓ Grape-fruit flavedo 90 % alcoholic extract, 0·5 cc. per head per day.
↓ Cod-liver oil, 3 drops per head per day.

Three standard piebald rats of 40 to 45 g. weight were restricted to a vitamin A-free diet, employing the usual Hopkins' basal ration of this laboratory. Vitamin D was supplied by direct irradiation of the animals, at a distance of 2 feet from a quartz mercury vapour lamp, for 10 minutes daily. At the end of 4 weeks growth had practically ceased. The fresh flavedo of ripe grape-fruit, finely minced and thoroughly mixed in with the basal ration, was then supplied at the level of 0·5 g. per head per diem. After the initial distaste of the animals had been overcome, practically the whole was eaten. A definite, though temporary, resumption of growth occurred. On the 49th day, when growth had ceased, the rind was substituted by an equivalent quantity (0·5 cc.) of the 90 % alcoholic extract. It was attempted to feed this orally by means of a hypodermic syringe, but the animals evinced strong dislike of the concentrated

bitter extract. Dilution with water had undesirable precipitation effects, besides increasing the amount to be administered. The extract was therefore well mixed in with the basal ration, and careful observation showed that the food intake was not decreased. Growth was resumed, but in the case of two animals proved only temporary, and on the 60th day of the experiment the third animal was also stationary. The three animals were all well below normal weight, and in two of them definite xerophthalmia had already developed. As it had now been clearly established that grape-fruit rind fed at this level was insufficient to protect the animals from xerophthalmia, and to sustain normal growth, cod-liver oil was supplied at the rate of three drops per head per diem. Vigorous growth was promptly resumed, and the xerophthalmia cleared up. On the 68th day of the experiment, when the animals appeared to be rapidly approaching normal, they were chloroformed and autopsied. No definite pathological changes could be observed, and it is worth while noticing that there was an appreciable amount of fatty tissue round the heart, indicating a satisfactory state of nutrition.

The result of this experiment, in which the animals were able to live and even grow slightly for 60 days on a diet in which the sole source of vitamin A was grape-fruit rind, seemed to indicate that the fresh flavedo of the grape-fruit contains small but definite amounts of this vitamin. The amount we had fed was only about a sixth of that given by Morgan, making allowance for the moisture in the fresh rind. In our experience it was impossible to induce the animals to take a larger dose, on account of the bitter taste of the material. It seemed desirable, therefore, to test the fresh rind oil, of which a proportionately larger dose could be given, assuming that the vitamin A is concentrated in the oil in grape-fruit rind, as Morgan and Chaney had shown it to be in orange rind.

Three standard albino rats were restricted to the same vitamin A-free diet. During this preparatory period one died, and its growth is therefore not shown in the figure. On the 29th day, when growth appeared to be held up, the oil extracted from the fresh rind was fed at the level of 0·1 g. per head per diem. A calibrated pipette was used, and as the oil had such a bitter taste that the animals would not tolerate its direct administration, it was mixed in with the basal ration. The food intake remained at about 10 g., and most of the oil was consumed. There was no growth response, and severe xerophthalmia developed in both animals. On the 48th day, when it became obvious that the two animals were suffering from definite vitamin A deficiency, cod-liver oil was fed at the rate of three drops per head per diem. The smaller animal, which was in the more severe condition, was chloroformed three days later. An autopsy showed severe xerophthalmia, enlarged suprarenals, and thinning of the gut. The other animal gradually recovered. Our conclusion is that grape-fruit rind oil contains at most only slight traces of vitamin A.

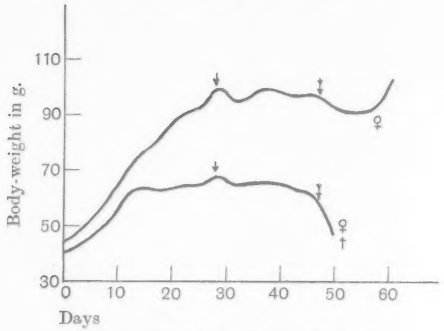

Fig. 2. Vitamin A of grape-fruit oil.
↓ Rind oil fed 0·1 g. per head per day.
↓ Cod-liver oil fed 3 drops per head per day.

Vitamin B.

Grape-fruit flavedo does not appear to have been previously investigated for vitamin B. In view of the finding of one of us [Willimott, 1926] that lemon flavedo contains considerable amounts of this vitamin, and also of the results reported by Morgan and Chaney [1924] that both orange and lemon peel contain it, it was thought of interest to examine the flavedo of the grape-fruit.

Four female standard albino rats of 54 to 64 g. weight were given a diet deficient in vitamin B. As it has been shown that lemon juice contains vitamin B [Bacharach, 1925], this addendum was omitted. It was not possible for the animals to obtain a supply of vitamin B by consumption of their faeces, as their cages were placed on trays into which the faecal matter fell out of their reach. On the 41st day of the experiment, growth in all four animals had ceased, the average weight being only 78 % of the normal. Evidences of the onset of paralysis were observed. Finely minced fresh grape-fruit flavedo, thoroughly mixed in with the basal ration, was now administered at the level of 0·5 g. per head per day. There was vigorous resumption of growth, and all symptoms of paralysis rapidly disappeared. On the 67th day, when the experiment was terminated, all four animals were in excellent condition, and growing rapidly. The average weight was about 90 % of the normal. From this experiment the conclusion was drawn that grape-fruit flavedo contains considerable amounts of vitamin B.

In view of the previous finding of one of us that 90 % alcohol would extract vitamin B from fresh lemon rind under certain specified conditions, it was thought of interest to test for this vitamin the alcoholic extract which we had prepared. A group of two male and one female rats was put on a vitamin B-free diet as before. After definite vitamin B deficiency had been established, 0·25 cc. per head per day of the alcoholic extract was administered. As this did not produce a marked average resumption of growth, the dose was increased to 0·5 cc., and later to 1·0 cc. The effect on growth was not strongly pronounced,

although no neuritic symptoms could be observed, and at the end of the experiment the animals appeared quite normal. It seemed from this result that the alcohol had not completely extracted the vitamin B from the rind tissue.

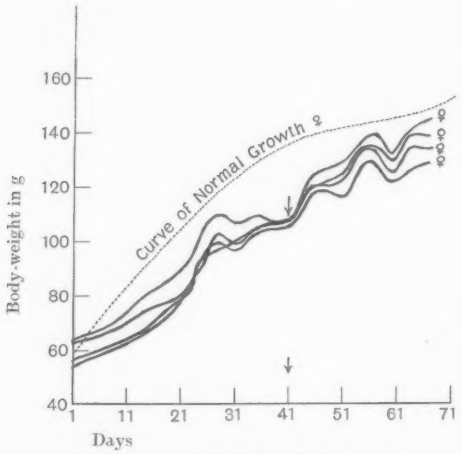

Fig. 3. Vitamin B of grape-fruit flavedo.
↓ 0·5 g. flavedo, fed per head per day.

Conclusions.

The results of our experiments show that grape-fruit rind contains little if any vitamin A, and that it gives a strong peroxidase reaction. Morgan and Chaney [1924], in commenting on the marked contrast in vitamin A content of both peel and juice between the three *Citrus* fruits, orange, lemon and grape-fruit, suggest that this may be related to the character of the lipochromes present. It is possible that light might be thrown on the problem by a study of the reaction and enzyme content of these three

Table I. *Vitamins, oxidising enzymes and reactions in ripe* Citrus *fruits.*

	Orange *Citrus aurantium*	Lemon *Citrus medica* var. *Limonum*	Grape-fruit *Citrus decumana*
A. Rind (flavedo)			
Vitamin content A	+ +	Slight	Very slight
,, ,, B	+ +	+ +	+ +
,, ,, C	+	Very slight	Not yet ascertained
p_H	4·4–4·8	4·2–4·4	4·3–4·6
Oxygenase reaction	–	–	–
Peroxidase reaction	+	+	+ +
B. Juice			
Vitamin content A	+ +	–	–
,, ,, B	+	+	+
,, ,, C	+ + +	+ + +	+ +
p_H	3·6–4·2	2·4–2·6	5·1–5·2
Oxygenase reaction	–	–	–
Peroxidase reaction	±	+	+

fruits. We have already published data on the reaction and tests for oxidising enzymes in the peel of the lemon at different stages of growth. This work has now been extended to the orange and grape-fruit. Samples of oranges and grape-fruits of different degrees of maturity are being received from California, and examined by the methods previously described [Willimott and Wokes, 1926]. In Table I are summarised data so far obtained by other workers and ourselves bearing on these points.

SUMMARY.

1. Grape-fruit rind contains considerable amounts of vitamin B, but practically no vitamin A. The content of vitamin C has not yet been investigated.

2. Naringin, the specific glucoside of the grape-fruit, was found present in the rind and in alcoholic extracts of it.

3. Data are presented on the reaction and content of oxidising enzymes as compared with the vitamin A content in the three *Citrus* fruits.

We desire to express our cordial thanks to Sir F. G. Hopkins for his support during the course of the work. We are also indebted to Dr T. Redman, of Liverpool University, for assistance in determining the p_H of the material.

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CLXIV. THE INFLUENCE OF THE COW'S DIET ON THE FAT-SOLUBLE VITAMINS OF WINTER MILK.

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In the early stages of research on vitamins Funk [1913] advanced the suggestion that the secretion of these factors in the milk was controlled by their presence in the food of the lactating animals. Subsequent investigations have proved this conjecture to be true. McCollum, Simmonds and Pitz [1916], working with rats, found that the development of the young was greatly influenced by the presence or absence of vitamin A or B in the diet of the nursing mothers. Hart, Steenbock and Ellis [1920], Dutcher and co-workers [1920], and Hess, Hunger and Supplee [1920] demonstrated that the anti-scorbutic potency of cow's milk depended on the antiscorbutic character of the diet of the cow, whilst Hughes, Fitch and Cave [1921], and Kennedy and Dutcher [1922] were responsible for proving that the vitamin A or B content of the milk was similarly influenced by the diet of the animals. The last of these investigators introduced vitamin A into the cows' ration in the form of alfalfa hay and corn silage. We [Drummond *et al.*, 1923, 1924] were able to confirm the observations of previous workers in this field, showing that by restricting the vitamin A content of the diet of the stall-fed cow a corresponding diminution of the vitamin takes place in the butter-fat. This fall in the vitamin A potency was shown by us to be rectified by the introduction of cod-liver oil into the deficient winter ration of the cows.

Apart from the direct evidence that the vitamin content of the milk of the cow is influenced by the diet of the animal, there is also some indirect evidence available supporting this view. In this connection one might quote the observations of Barnes and Hume [1919] on the seasonal variations in the antiscorbutic properties of cow's milk and those of Drummond, Coward and Watson [1921] on the variation in the amount of vitamin A in butter. Although a few investigators were unable to confirm this influence of diet on the vitamin content of the milk, the weight of evidence obtained from so many independent and careful investigations leaves no doubt as to its reality.

All the experiments referred to above were carried out at a time when the

separate identity of the antirachitic vitamin (vitamin D) and of vitamin A was not fully appreciated and consequently the "Vitamin A" measure of the cows' diet and that of the milk obtained from these animals as determined by these earlier researches give us no idea of the relative proportions of the two fat-soluble principles present in either.

The discovery of McCollum, Simmonds, Shipley and Park [1922, 1, 2, 3] that the two "fat-soluble" vitamins were not identical was soon corroborated by various workers and it became apparent that certain green vegetables did not contain much of the antirachitic vitamin [McClendon and Schuck, 1923; Zucker, 1923; Goldblatt and Zilva, 1923; Chick and Roscoe, 1926; Boas, 1926]. The experiments of Goldblatt and Zilva made the low calcifying potency of spinach as compared with cod-liver oil particularly plain. They found that whilst a daily dose of 0·1–0·25 g. of spinach promoted growth in rats on a diet deficient in the fat-soluble vitamin, a dose of 3 g. did not have the slightest effect in promoting calcium deposition, although 2·2–4·5 mg. of cod-liver oil was found sufficient in this respect. If, therefore, other green fodder behaved similarly to spinach, the milk from winter cows fed on a basal ration supplemented with green vegetables would be poor in the antirachitic vitamin in spite of its growth-promoting properties. On the other hand, the milk from the cows which received an addition of cod-liver oil with their winter ration might yield a milk of enhanced antirachitic potency. Recent work [Luce, 1924; Chick and Roscoe, 1926] has shown that the presence of green fodder in the ration of the cow increases the vitamin A content of the milk, whereas it has only a slight effect on the antirachitic factor.

In attempting to test this hypothesis experimentally we were faced with some technical difficulties. In our first effort in the winter of 1924–25 we employed the old growth method of Zilva and Miura [1921] for the estimation of vitamin A. In view of important work on this subject by Steenbock and his collaborators [1923, 1924] growth cannot be accepted as a reliable criterion for the assay of vitamin A unless other vitamins are available in adequate amounts. These workers have shown that each fat-soluble vitamin is able to promote growth for as long a time as the other is present as a reserve in the animal, and consequently the interpretation of the results obtained in our first experiments was not warranted, although there were definite indications that our surmise as regards the influence of the green fodder and cod-liver oil of the cows' ration on the milk was justified. The work was therefore repeated in the winter 1925–26. In this effort we were greatly aided by the experiences of the preceding year. Above all, we had the advantage of utilising for our tests a standardised breed of young rats which are now specially bred at the Lister Institute for use in the estimation of the fat-soluble vitamins [Smith and Chick, 1926]. Sources of error such as the variation in the reserves of these two vitamins in the experimental animals were thus eliminated. The importance of this has been pointed out by Chick [1926] in her critical study on the biological methods for the estimation of the fat-

soluble vitamins. Vitamin A was in the present investigation estimated by a method based on Steenbock's principles, namely, by administering the butters to rats which were declining in weight on a diet free from fat-soluble vitamins but containing irradiated hardened cotton-seed oil as a source of vitamin D.

This final set of experiments produced definite evidence that cows receiving a supplement of green vegetables (kale) with their stall diet yield a butter rich in vitamin A but poor in the antirachitic vitamin, whilst cows receiving a supplement of cod-liver oil produce a milk rich in both fat-soluble vitamins. In spite of their superior vitamin A content the antirachitic value of the butters from the cows receiving kale was found to be of the same magnitude as that of the butters from the cows receiving the basal diet without supplement which was a diet altogether free from fat-soluble vitamins. The significance of this fact from the hygienic point of view will be discussed later.

Experimental.

Six dairy shorthorn cows from the herd of the National Institute for Research in Dairying at Reading were employed in the 1925–26 experiment. The animals were divided into three groups but the milk of each individual cow was kept separate. The groups were arranged as follows:

Table I.

		Last calved	No. of calves	Weight 19. i. 26		Weight 13. iv. 26	
Basal diet	Lily II	9. x. 25	1	8 cwts.	97 lbs.	9 cwts.	55 lbs.
,,	Welcome	7. x. 25	3	10	105	11	52
Basal diet and kale	Bertha	5. xi. 25	3	9	111	9	104
,, ,,	Firefly II	24. ix. 25	2	10	32	10	43
Basal diet and cod-liver oil	Rosemary	6. xi. 25	8	10	92	10	54
	Doll II	1. xi. 25	2	9	64	9	79

The cows were stalled at the end of November. From the first week in December their diet consisted of mangolds, oat straw and meal mixture. The experiment started on Feb. 4th. The cows on the basal rations, Lily and Welcome, received oat straw, roots, and meal mixture[1]. Bertha and Firefly received kale instead of roots, whilst Rosemary and Doll received the basal ration of oat straw, roots and meal mixture supplemented by a daily dose of cod-liver oil[2]. The cod-liver oil dose was started at 2 oz., increased after 8 days to 4 oz., after another 19 days to 6 oz., and the final dose of 8 oz., which was administered after a further interval of 21 days, was continued to the end of the experiment in the middle of April. The oil was given with the ration and was always consumed quantitatively. The consumption of food by the cows

[1] The meal mixture was composed of two parts of extracted palm kernel meal, two parts of bean meal, one part of decorticated extracted ground nut meal, one part of crushed barley, one part of crushed wheat and of a little steamed bone flour and calcium carbonate.

[2] The cod-liver oil, as tested by the technique to be described later, was found to contain vitamins A and D.

per day towards the end of the experiment represented approximately the average consumption and was as follows:

Table II.

Food	Lily II	Firefly II	Welcome	Doll II	Bertha	Rosemary
Straw	10 lbs.	7 lbs.	10 lbs.	8 lbs.	7 lbs.	10 lbs.
Roots	80	—	80	60	—	80
Kale	—	40	—	—	40	—
Cod-liver oil	—	—	—	8 ozs.	—	8 ozs.
Meal mixture	10	11	14	15 lbs.	14	14 lbs.

The cows were kept in well-ventilated stalls where the access of light was very poor and exposure to direct sunlight was excluded. The animals were milked twice a day, at about 7 a.m. and 4 p.m., and their walk from their stalls to the milking sheds was their only exercise. Water was laid on for each cow.

About the middle of April the butters were prepared from the respective milks for testing purposes. All the milk from four consecutive milkings on two days was employed for the butter of each cow. After separating the milk, the cream was ripened by a special starter prepared in separated milk. When the ripening was complete the butter was churned in a 2-minute churn, washed in the usual way with water and made up. Precautions were of course taken to prevent the butter of one cow from coming into contact with that of another in the churning process. The butters from the cows on basal diet were white and those from the "cod-liver oil" cows were equally colourless and did not possess a "fishy" flavour or any other taint. The butters from the kale-fed cows, especially from Firefly, possessed the usual yellow colour. The vitamin determinations which were carried out at the Lister Institute began early in May. During the period which elapsed between the preparation and the termination of the testing, the end of June, the butters were kept in the dark in cold store.

There was a very marked drop (about 30 %) in the percentage of fat of the milk from the cows receiving cod-liver oil when the dose was increased to 8 oz. The percentage of the fat in the milk from the cows fed on kale on the other hand remained the same as in that obtained from the control cows. This phenomenon was previously observed by us [Drummond *et al.*, 1924, Channon *et al.*, 1924] as well as by Wagner and Wimberger [1925]. The reduction in the *actual* amount of fat produced by the "cod-liver oil cows" per day was not, however, as great as it might appear from the percentage figures. This is seen from Table III in which the yield of milk-fat per day (an average of three successive days) at the commencement and at the end of the experiment from each cow is given.

The figures show that there was a diminution in the daily yield of milk-fat in all the cows due to the natural fall in the quantity of milk as the lactation period advances. The "cod-liver oil cows," however, showed a decidedly lower production of milk-fat per day. If the average fall in yield of fat by the other cows be taken into account the extra diminution amounted to about 15 %.

Table III.

Diet and cow	Dates	Average daily milk yield lbs.	Average daily fat yield lbs.	Drop in fat yield %	Mean drop in fat yield %
Basal diet + cod-liver oil					
Doll II	Feb. 1st, 2nd, 3rd	28	1·299	32·9	29
	Apr. 10th, 11th, 12th	26	0·872		
Rosemary	Feb. 1st, 2nd, 3rd	27	1·131	25·1	
	Apr. 10th, 11th, 12th	29	0·847		
Basal diet					
Lily II	Feb. 1st, 2nd, 3rd	17	0·699	8·4	14
	Apr. 13th, 14th, 15th	15	0·640		
Welcome	Feb. 1st, 2nd, 3rd	25	1·001	17·9	
	Apr. 12th, 13th, 14th	20	0·822		
Basal diet + kale					
Firefly II	Feb. 1st, 2nd, 3rd	20	0·906	17·7	
	Apr. 13th, 14th, 15th	17	0·746		
Bertha	Feb. 1st, 2nd, 3rd	22	1·180	15·4	
	Apr. 10th, 11th, 12th	21	0·998		

The influence of cod-liver oil on the milk-fat yield of the cow will form the subject of another communication.

Vitamin A was estimated by a method which has been recently used at the Lister Institute with success [see Chick and Roscoe, 1926] similar to that described by Steenbock, Nelson and Black [1924]. Young rats about 4 weeks old and weighing about 35 g. were placed on the diet deficient in fat-soluble vitamins used by Zilva and Miura [1921] and made up as follows:

Heated caseinogen	20 g.
Starch	50 g.
Hardened cotton-seed or sesame oil	15 g.
Marmite	5 g.
*Salt mixture	5 g.
Decitrated lemon juice	5 g.
Water	50 g.

* No. 185, MacCollum, Simmonds and Pitz [1917].

When the rats on the above diet ceased to grow in weight after 3–4 weeks, the hardened cotton-seed oil was replaced by a similar oil previously irradiated for 30 minutes at a distance of 33 cm. from a quartz mercury lamp. Growth was then restored for another 3–4 weeks when the second decline in weight due to the vitamin A deficiency set in. The butters were then administered. Doses of 0·2 g. and 0·6 g. from each butter were given daily, care being taken to ensure the total consumption of the doses. As a negative control a group of rats were left on the basal ration containing the irradiated oil only. Another group of rats receiving cod-liver oil served as a positive control. The degree of growth was taken as a criterion for the vitamin A assessment.

The antirachitic potency of the butters was arrived at by feeding them to rats subsisting on McCollum's 3143 rickets-producing diet low in phosphorus. Rats about a month old were placed on this diet supplemented by the butter doses. In this case daily doses of 0·2 g. and 0·6 g. of each butter were also used. The group of rats serving as a negative control received only the basal diet,

whilst some of the animals in the positive control group received one drop, others three drops of cod-liver oil. After 28 days the animals were killed and the femur, tibia, and fibula were dissected and carefully cleaned of all adhering flesh and weighed. The dry weight was obtained after drying the bones for 24 hours at 110°. The dry bones were then wrapped up in finely meshed silk material, broken up and extracted with boiling alcohol for 8 hours and subsequently with ether for 16 hours, after which they were removed to weighing bottles, dried again at 110° for 2 hours and weighed. The ash content of the bone was obtained by incineration to constant weight.

The main difference in composition of the bones between rats receiving a sufficient dose of cod-liver oil as an addition to the above diet and those maintained on the experimental diet alone lies in the water content and in the ash content of the bone. Chick, Korenchevsky and Roscoe [1926] suggest a convenient standard of comparison, namely, the ratio A/R of the ash in the bone to the organic material other than substances extracted by hot alcohol and ether. We found this standard to be a reliable index of the degree of calcification and very convenient for comparative purposes.

Vitamin A. Three rats were utilised for each dose of butter. In this experiment about 50 rats of approximately the same age and reared under the same conditions had to be collected. Consequently, it was difficult to prevent the inclusion of a few animals which did not come up to the high standard of the physical condition of the majority of the group. Any suspicious animals in this respect were placed in the group of the positive control, the animals of which group received cod-liver oil daily. Some of the delicate animals died in the early stages of the experiments. The remainder, as will be seen from Table IV, in spite of their condition, responded well to the addition of vitamin A in the form of cod-liver oil to the otherwise complete diet. Negative controls which received no vitamin A lost in weight and eventually succumbed as a result of this vitamin deficiency. The rats receiving the butters from the cows on the basal diet (Lily and Welcome) showed hardly any growth on the 0·2 g. dose and some growth on the higher dose of 0·6 g. The butter from the cow Welcome was decidedly worse than that from her fellow on the same diet. When we turn to the group of rats fed on the butters from the cows which received kale in addition to the basal diet (Bertha and Firefly) we find a decidedly higher growth-promoting activity in this butter as compared with that from the cows on the basal ration. This activity is still more marked in the butters from the cows whose ration was supplemented by cod-liver oil. These results are represented graphically in Figs. 1 and 2, in which the average increase in weight is plotted against time. This experiment leaves little doubt that the addition of kale or cod-liver oil to the basal ration of cows improves the vitamin A content of the butter.

Vitamin D. Detailed results of these estimations are given in Tables IV and V, a summary of the averages of these figures in Table V, whilst Figs. 1 and 2 give a graphic representation of the average values of the A/R ratio

Table IV.

Rat	Initial weight g.	Final weight g.	Weight of bone g.	% of water	% of fat	% of organic residue	% of ash	Ratio A/R
			A. Negative controls					
658 ♀	34	51	0·7835	66·79	2·49	23·74	6·98	0·294
669 ♀	40	63	0·8892	66·44	1·96	23·61	7·98	0·338
678 ♀	48	65	0·9742	64·98	4·95	21·89	8·17	0·373
689 ♂	51	83	1·1235	64·57	3·51	22·62	9·30	0·411
698 ♀	44	65	0·9720	64·07	5·12	22·10	8·71	0·394
706 ♂	46	59	0·8990	64·07	3·77	22·39	9·77	0·436
712 ♀	42	69	0·9340	62·15	4·39	22·49	10·97	0·488
724 ♂	48	63	0·9878	66·18	3·86	22·63	7·33	0·323
			B. Positive controls One drop cod-liver oil					
668 ♀	39	47	0·7939	57·74	4·95	21·67	15·64	0·722
677 ♀	43	56	0·8305	56·68	5·74	21·39	16·19	0·757
688 ♂	36	42	0·7195	—	—	21·64	13·27	0·613
697 ♀	41	62	0·9985	57·93	6·39	21·58	14·10	0·653
705 ♂	37	54	0·8070	59·23	4·97	23·20	12·60	0·543
			Three drops cod-liver oil					
657 ♀	32	44	0·7190	60·15	5·01	20·93	13·91	0·664
711 ♀	37	48	0·7561	55·14	5·99	21·93	16·94	0·773
723 ♂	40	53	0·8021	—	—	21·76	16·76	0·770
			C. Basal ration (Lily) 0·2 g. butter					
670 ♀	45	59	0·8859	64·45	3·99	23·37	8·19	0·352
649 ♀	36	49	0·7839	67·17	2·85	22·97	7·01	0·306
663 ♂	46	74	1·0993	66·86	3·87	22·33	6·94	0·311
679 ♀	42	66	0·9570	60·08	8·64	23·31	7·97	0·342
690 ♂	43	60	0·9605	63·61	5·49	21·96	8·94	0·408
713 ♂	44	66	0·9366	63·91	3·01	22·82	10·26	0·450
			0·6 g. butter					
654 ♂	35	40	0·7782	66·19	4·70	20·88	8·09	0·388
685 ♀	41	60	0·8833	63·84	3·44	22·53	10·19	0·452
694 ♀	42	56	0·8680	60·83	6·54	21·29	11·34	0·532
717 ♀	41	58	0·8790	58·68	4·31	21·82	15·19	0·665
			D. Basal ration (Welcome) 0·2 g. butter					
650 ♂	39	63	0·9126	67·29	2·28	23·31	7·12	0·305
664 ♀	42	63	0·9524	65·07	2·46	23·49	8·98	0·382
671 ♀	46	70	1·0017	65·21	5·18	21·83	7·78	0·357
680 ♂	44	67	1·0821	65·75	2·85	22·91	8·49	0·370
699 ♀	37	47	0·7155	61·58	5·71	22·59	10·12	0·448
707 ♂	42	61	0·9058	60·38	4·45	22·75	12·42	0·543
			0·6 g. butter					
660 ♀	42	56	0·8610	63·94	4·86	21·71	9·49	0·436
674 ♂	45	60	0·9105	59·21	6·59	21·91	12·29	0·643
702 ♀	36	45	0·7440	60·40	7·20	21·75	10·65	0·489
718 ♀	37	47	0·7661	61·74	2·86	23·23	12·17	0·523
			E. Kale (Bertha) 0·2 g. butter					
651 ♀	37	52	0·8222	66·78	3·61	22·61	7·00	0·310
665 ♂	43	60	0·9856	66·82	4·69	23·59	4·90	0·208
681 ♀	42	58	0·9320	63·63	3·63	23·35	9·39	0·402
691 ♂	44	55	0·9465	57·95	8·11	23·20	10·74	0·463
708 ♀	42	56	0·8650	59·77	7·32	22·66	10·25	0·452
714 ♂	43	57	0·8794	62·54	5·93	23·34	9·19	0·391
			0·6 g. butter					
655 ♂	34	41	0·7866	66·10	4·53	21·26	8·11	0·381
686 ♀	39	59	0·8465	63·22	3·53	22·67	10·58	0·467
695 ♀	42	53	0·8255	57·12	8·72	21·58	12·58	0·582
719 ♀	38	58	0·8670	60·55	5·13	21·57	12·75	0·591

Table IV (*cont.*).

Rat	Initial weight g.	Final weight g.	Weight of bone g.	% of water	% of fat	% of organic residue	% of ash	Ratio A/R
			F. Kale (Firefly II)					
			0·2 g. butter					
652 ♀	35	54	0·7990	66·64	3·89	21·96	6·89	0·312
666 ♀	44	57	0·8861	65·28	3·04	23·76	7·92	0·333
672 ♂	50	66	0·9970	63·65	6·28	22·19	7·88	0·355
682 ♂	45	63	1·0265	63·84	3·50	22·83	9·83	0·431
700 ♀	37	45	0·7890	63·13	4·03	23·39	9·45	0·404
715 ♂	41	59	0·8940	62·03	4·86	21·87	11·24	0·511
			0·6 g. butter					
661 ♀	42	58	0·8935	65·78	4·63	21·13	8·46	0·401
675 ♀	44	47	0·9003	61·03	7·17	21·25	10·55	0·497
703 ♂	36	47	0·7860	62·21	5·35	21·44	11·00	0·514
720 ♂	40	47	0·7741	53·76	10·67	21·84	13·73	0·639
			G. Cod-liver oil (Rosemary)					
			0·2 g. butter					
653 ♀	34	55	0·8432	67·06	2·95	22·46	7·53	0·335
667 ♂	42	69	1·0238	66·90	4·22	20·88	8·00	0·384
683 ♀	41	59	0·9530	63·55	3·93	22·00	10·52	0·465
692 ♀	43	65	0·8730	60·57	6·83	21·94	10·66	0·486
709 ♀	37	47	0·7695	59·71	12·13	18·23	9·93	0·541
716 ♂	40	59	0·8720	62·21	4·24	21·62	11·93	0·639
			0·6 g. butter					
656 ♂	36	63	0·9273	62·89	4·84	20·30	11·97	0·590
687 ♀	37	43	0·8120	59·17	4·47	21·64	14·72	0·680
696 ♀	42	55	0·8835	57·55	7·91	21·43	13·11	0·612
721 ♂	40	57	0·8619	56·66	6·97	21·40	14·97	0·699
			H. Cod-liver oil (Doll II)					
			0·2 g. butter					
659 ♀	44	68	0·9336	64·53	4·69	21·19	9·59	0·452
673 ♀	47	68	0·9480	63·05	5·78	21·23	9·94	0·469
684 ♂	45	59	0·9855	63·12	3·93	22·07	10·88	0·473
693 ♀	43	54	0·8690	60·64	6·59	22·47	10·30	0·459
701 ♂	43	56	0·9150	62·18	3·71	22·36	11·75	0·526
710 ♂	41	67	0·9430	60·77	6·20	21·95	11·08	0·505
			0·6 g. butter					
662 ♀	42	64	0·9453	64·05	4·12	21·13	10·70	0·507
676 ♂	44	54	0·8657	56·67	7·73	19·94	15·66	0·785
704 ♀	34	47	0·7560	60·85	5·79	19·97	13·39	0·669
722 ♀	36	43	0·7338	54·75	7·56	21·34	16·35	0·767

obtained with rats fed on the butters from the different cows. It is seen that the rats receiving 0·2 g. of butter from the basal cows and from the kale-fed cows behaved very much like the negative control rats receiving no butter supplement in their diet. On the other hand, the same dose of butter from the "cod-liver oil cows" showed a definite antirachitic potency (Fig. 1). When the doses of the butters given to the test rats were trebled (Fig. 2) the butter from the basal and kale-fed cows showed an antirachitic potency of the same magnitude. This potency was, however, decidedly lower than that shown by the butters from the "cod-liver oil cows." The latter butters were almost as protective in this dose as the one drop of cod-liver oil given to some of the animals of the positive control rats. As there was only a small difference in the behaviour of the two sets of animals in the positive control group, one may conclude that the maximum standard of calcification possible to demonstrate

Table V.

Diet and cow	Dose of butter g.	% H_2O		% fat		% organic residue		% ash		Ratio A/R	
Basal											
Lily	0·2	64·35	64·28	4·64	4·23	22·79	22·76	8·22	8·69	0·368	0·385
Welcome	,,	64·21		3·82		22·73		9·15		0·401	
Lily	0·6	62·77	61·55	4·75	5·07	21·63	21·89	11·20	11·18	0·509	0·516
Welcome	,,	61·32		5·38		22·15		11·15		0·523	
Kale											
Bertha	0·2	62·92	63·51	5·55	4·91	23·13	22·90	8·58	8·73	0·371	0·381
Firefly II	,,	64·10		4·27		22·67		8·87		0·391	
Bertha	0·6	61·75	61·23	5·48	6·22	21·77	21·60	11·01	10·98	0·505	0·509
Firefly II	,,	60·70		6·96		21·43		10·94		0·513	
Cod-liver oil											
Rosemary	0·2	63·33	62·86	5·71	5·43	21·19	21·53	9·76	10·18	0·475	0·478
Doll II	,,	62·38		5·15		21·86		10·60		0·481	
Rosemary	0·6	59·07	59·08	6·05	6·18	21·19	21·15	13·69	13·85	0·645	0·639
Doll II	,,	59·08		6·30		21·10		14·00		0·632	
Negative controls	—	64·89		3·76		22·68		8·65		0·382	
Positive controls	1 drop	57·89		5·51		21·90		14·36		0·658	
	3 drops	57·65		5·50		21·54		15·87		0·735	

by this method has been almost reached by the administration of one drop of cod-liver oil.

In comparing the results of the vitamin D tests of the butters with those of the vitamin A tests it becomes evident that whilst the addition of kale to the basal ration raises the vitamin A content of the milk it does not contribute to the enhancement of its antirachitic potency. In the case of the "cod-liver oil cows" there is a marked augmentation in the potency of both vitamins.

In our experiments the water content was higher and the ash of the bones lower than in those of Chick, Korenchevsky and Roscoe [1926]. This is most probably due to the fact that the batch of rats employed by us had lower initial reserves of the antirachitic vitamin. Our results, however, fully confirm the observation of these workers. There is a decided higher water and lower ash content in the bones of the negative control animals as compared with the positive control group. The corresponding differences in fat content and the "organic residue" are also similar. It will be seen that the value of the ratio of ash to "organic residue," as suggested by the above workers, serves as a very convenient index of the antirachitic protection effected by substances incorporated in the basal diet.

The comparative superiority of the calcium-depositing properties of butters from cows receiving cod-liver oil over those of butters from kale-fed cows also emerges from the results we obtained in our 1924–25 experiments. The test rats were fed on a basal diet complete in every respect except the fat-soluble vitamins, and growth was observed by the method of Zilva and Miura and the mineral ash content of the bones of the test rats estimated after a period of 7 weeks on the experimental diet to which small daily rations of the butter were added. The butters were tested on three different occasions during the period in which the cows were on the experimental diets. In the third test the

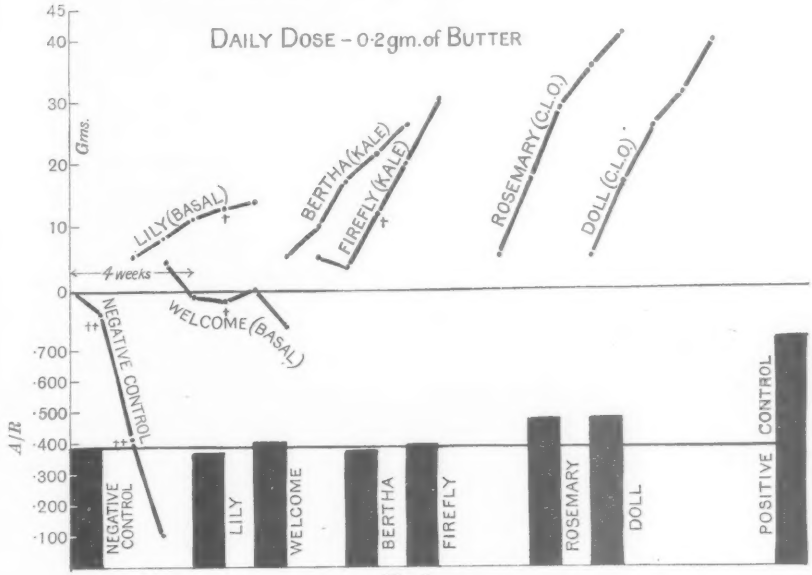

Fig. 1.

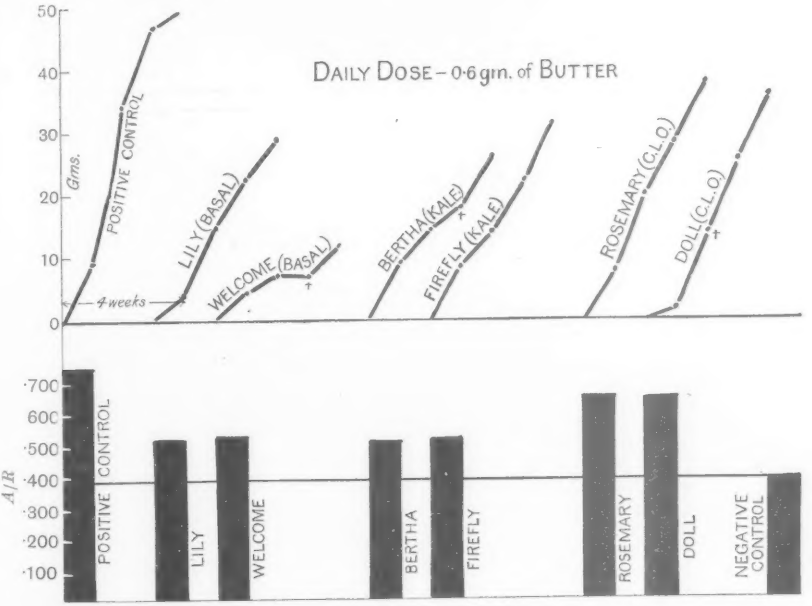

Fig. 2.

Figs. 1 and 2. The curves show the average increase in weight of the test rats. The preliminary period is omitted. Individual deaths in the groups are marked by a cross.

results were most marked, since by then the reserves of the antirachitic vitamin of the cows were run out and consequently the influence of the antirachitic character of the diet on the milk was most pronounced. In Table VI only the averages of the percentages of ash in the fat-extracted bone from the rats on the various butters in the third test are given. Three doses of butter were used in this investigation, namely, 0·2 g., 0·4 g., 0·8 g. The last dose, however, was found to be too high and had a disturbing effect on the rats, for which reason we adopted 0·2 g. and 0·6 g. as test doses in the 1925–26 investigation.

From Table VI it is seen that whilst the percentages of the ash content of the bones from the rats receiving the butters from the basal diet cows and from

Table VI.

Cow and diet	% of ash in dry extracted bone: 0·2 g. butter		0·4 g. butter	
Basal diet				
Bertha	52·27	52·52	54·55	53·52
Carlet	52·77		52·58	
Basal diet and kale				
Firefly II	52·99	53·12	51·07	52·58
Welcome	53·25		54·09	
Basal diet and cod-liver oil				
Doll II	55·94	56·24	55·21	56·08
Winsome	56·54		56·96	
Negative controls	51·90 (only basal diet)		—	
Positive controls	59·16 (3 drops of cod-liver oil)		—	

the kale-fed cows are the same and almost of the same magnitude as the negative controls, the percentage of the ash in the group of rats on the "cod-liver oil butter" is definitely higher. There is no differentiation between the behaviour of the animals on the 0·2 g. dose and those on the 0·4 g. dose. This we can only explain by the fact that the less vigorous rats of the available supply were used purposely in testing the higher dose. The results of this experiment, however, when taken in conjunction with those of the 1925–26 investigation indicate an improvement in the antirachitic potency of the "cod-liver oil butters" as compared with the butters from the kale-fed cows and with those from the cows on the basal diet.

Conclusion.

Taking the A/R ratio in the bones of our test animals as the basis of comparison for the antirachitic potency of the different butters it is seen that the antirachitic value of the milk varies with the increase in the A/R ratio above the figures obtained with the negative controls. By comparing these variables it is found that the antirachitic potency of the "cod-liver oil butters" is twice as high as that of the butters from the kale-fed cows and from the cows fed on the basal ration. An increase of this magnitude can only be explained by

a passage of the antirachitic vitamin of the cod-liver oil into the milk. One must of course consider the possibility of an increase in the vitamin content of the butter owing to the decreased yield of butter-fat brought about by the administration of the cod-liver oil. Such an increase, however, could, as we have seen, only be of the order of 15 %. Experiments with smaller doses of cod-liver oil will throw further light on this aspect of the question.

The passage of the antirachitic vitamin from the diet into the milk of the lactating animal cannot be looked upon as an unexpected result when one considers the analogous behaviour of the other vitamins in this respect. The work of McCollum, Simmonds and Pitz [1916] on rats, mentioned in the introductory part of this communication, Miss Hume's investigations at Vienna on human milks [1923], Korenchevsky's [1921, 1923, 1925] contributions on the influence of the presence of the antirachitic vitamin in the diet of the pregnant and lactating rat on the progeny, all pointed to the passage of the antirachitic principle from the diet into the milk of the mother.

By observing the effect upon children suffering from florid rickets Wagner and Wimberger [1925] studied clinically the relative antirachitic potency of milks from cows receiving cod-liver oil in their ration and from cows subsisting on a basal diet. Parallel laboratory tests were carried out on the milks used by Wagner and Wimberger by Dr E. M. Luce at the Lister Institute with the object of ascertaining the relative vitamin A and vitamin D activity of the samples of milk. In view of the fact that the growth-promoting test was employed in the estimation of vitamin A these results may not be correctly interpreted, but the antirachitic tests, in spite of the small number of rats employed, and the rather doubtful basal diet of the cows (clover, brewer's grains, clover hay and bran) show that the "cod-liver oil" milks were definitely superior to the control milks. Thus for test rats on a dose of 5 cc. of milk the percentage of calcium in dry bone (not extracted) was 16·5 for the "cod-liver oil milk rats" as compared with 14·1 for the "control milk rats." This differentiation was rather less marked with the higher dose of 10 cc., probably because of the appreciable antirachitic potency of their control milk. The cure of florid rickets was presumably too severe a test and influenced by the negative results which they obtained, Wagner and Wimberger, to our mind, discount the suggestive nature of the results on rats when they say "Im Zusammenhang mit den Versuchen am menschlichen Säugling aber darf man auch dieser geringen Überlegenheit der 'Lebertranmilch' keinen zu grossen Wert beilegen."

Our results are not without practical significance when we consider that infants born during the winter and brought up on cow's milk would depend almost entirely, unless cod-liver oil were given, for their supply of the antirachitic vitamin on the milk, during the early months of their existence. The antirachitic value of the average winter milk would not be of a much higher order than that of the milk from our basal- or kale-fed cows. The antirachitic potency of such milk, it appears from our results, can be doubled by administering cod-liver oil to the cows during the winter months. Several points have

still to be cleared up. It is of importance to ascertain whether lower doses of cod-liver oil, such as do not depress the yield of milk fat, will enhance the antirachitic potency of the milk to the same extent as the higher doses. Also whether the administration of cod-liver oil to the cows during the summer months could raise the antirachitic activity of their milk in summer also, and lastly to obtain clinical confirmation of the above laboratory observations. The latter task is fraught with many difficulties, but should not be insuperable in spite of the negative results so far obtained. The clinical confirmation of the laboratory work in connection with the other vitamins encourages one to think that a parallelism also exists in this case.

Summary.

(1) The inclusion of kale in the winter ration of the cow raises the vitamin A content but not the vitamin D content of the milk.

(2) The inclusion of cod-liver oil in the winter ration of the cow raises the vitamin A and the vitamin D content of the milk.

(3) Previous observation that the administration of high doses of cod-liver oil to cows reduces the percentage of fat in their milk is confirmed.

We wish to express our best thanks to Mr James Mackintosh for supervising the management and feeding of the cows, to Miss E. M. Hallum for preparing the butters and to Miss J. R. L. Rennie for general assistance.

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CLXV. THE DEGRADATION OF GLUCOSE BY THE BLOOD CORPUSCLE OF THE RABBIT. II.

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In a previous communication [Irving, 1926] evidence was adduced on which was based the theory that the glucose which was degraded by blood corpuscles during glycolysis was so changed at the surface of the cell. In the present paper, the writer has further investigated the mechanism involved, and presents data relating to the intervention of organic phosphorus, the influence of ions, and the degradation of some stereoisomers of glucose.

Experimental.

The technique employed was precisely the same as that described in the previous communication, save that in some of the experiments with large quantities of blood, the mixed arterial and venous blood of one or more rabbits was employed. The same precautions as regards bacterial contamination were taken. Very few of the experiments lasted more than four hours.

The intervention of phosphorus.

Martland and Robison [1924] have shown that after haemolysis of the blood corpuscles free phosphorus is rapidly liberated from the pre-existing organic phosphorus compounds in these cells. Piazza [1925], and Morgulis and Barkus [1925] stated that glycolysis had no relationship with this hydrolysis since it was absent or slight when the production of free phosphorus was most marked. The same was found by Martland, Hansman and Robison [1924] and has been confirmed by the present writer.

If the corpuscles were kept in a strictly isotonic medium, the liberation of free phosphorus was very slight and the conditions for glucose degradation were at an optimum, but if haemolysis occurred, free phosphorus was liberated and glycolysis depressed. It appeared that perhaps the "intactness" of the organic phosphorus compounds might be essential for glucose degradation, but since both these phenomena depend on the integrity of the cell for their occurrence, it is more probable that this is the essential factor in both cases.

Bierry and Moquet [1925] stated that during the early stages of glycolysis the free phosphorus in blood fell in amount, and that this indicated the temporary synthesis of an organic phosphorus compound, which was an intermediate step in the reaction. The present writer undertook several experiments in order to test this hypothesis.

Owing to the fact that phosphorus estimations in suspensions of corpuscles during glycolysis are valueless after about the first hour, because of the rapidly increasing liberation of free phosphorus from the preformed organic phosphorus compounds, the experiments described were usually terminated after an hour. The following is typical of several.

A rabbit was killed and the mixed arterial and venous blood collected and centrifuged in the usual way. The plasma was removed and to a given volume of corpuscles an equal volume of a very dilute phosphate mixture in isotonic saline was added. The resulting suspension was divided into two portions, (1) to act as a control, and (2) to which glucose was to be added. The free phosphate and glucose were each estimated in both tubes, the former by the Briggs [1922] technique. A weighed amount of glucose was then added to (2) and both tubes were corked and well shaken. The free phosphorus content of both tubes, and the glucose content of (2) were again estimated. They were then placed in the incubator at 37°, and the free phosphorus content of both tubes and the glucose content of (2) estimated at frequent intervals. The results are shown in Table I.

Table I.

	Tube (1)		Tube (2)		
Hours	mg. P %	Glucose %	mg. P %	Glucose %	Remarks
—	6·77	0·059	6·57	0·059	(Preliminary).
0·0	6·65	—	6·59	0·197	Glucose added to (2)
0·25	7·01	—	6·79	—	Incubation at 37°
0·5	7·92	—	7·24	—	
1·0	—	—	—	0·177	
2·5	—	—	—	0·152	

As will be seen, the addition of glucose to (2) did not alter in any way its free phosphorus content. This remained steady in both tubes till after about half an hour it began to rise under the influence of the corpuscular esterase. Thus the addition of glucose does not seem to lead to the formation of an organic phosphorus compound.

The results of Bierry and Moquet may perhaps be ascribed to the shaking out of CO_2, incidental to drawing, rendering the blood slightly more alkaline and thus starting the synthetic action of the phosphoric esterase mechanism described by Martland [1925]. The present writer has been able to obtain a fall in the free phosphorus by this means. The slight change in free PO_4 in tube (1) in Table I at 0 hours could only be ascribed to shaking out of CO_2.

The influence of initial low concentrations of free phosphorus was now tested. Rabbit's corpuscles were twice washed with isotonic saline and divided

into two portions. One was suspended in an equal volume of 1/750 hydroxyquinoline sulphate, and the other in an equal volume of a dilute isotonic phosphate mixture in presence of antiseptic. Glucose was added to both and the initial free phosphate estimated before incubation. The results of one experiment are shown in Table II.

Table II.

	Tube (1)			Tube (2)		
Hours	Glucose %	Glucose degraded %	mg. P %	Glucose %	Glucose degraded %	mg. P %
0	0·127	—	>0·5	0·108	—	6·85
2	0·097	0·030	—	0·081	0·027	—
4	0·074	0·053	—	0·057	0·051	—

In other experiments, the washing sometimes had a slowing influence on the rate of glucose degradation [cf. Macleod, 1913]; this was not however due to washing away of inorganic phosphorus since the subsequent addition of phosphates did not have any accelerating action.

Experiments in which the action of corpuscles on potassium hexosediphosphate was tested, indicated that, as far as the reducing power of the solution was concerned, this ester was very slowly attacked. It would thus appear improbable that it was an intermediate step in the conversion of glucose to lactic acid by these cells.

The evidence adduced above can therefore be summarised as follows.

The preformed organic phosphorus compounds in the corpuscles have no influence on glycolysis in blood. The addition of glucose to a corpuscular suspension at room temperature or at 37° does not bring about a diminution in the amount of free phosphorus present, nor has the initial concentration of the latter (within the limits of estimation of the methods employed) any appreciable influence on the rate of glycolysis. It appears unlikely that hexosediphosphate is an intermediate metabolite.

The influence of ions.

Hydrogen ion concentration. Several workers have stated that increased acidity retards glycolysis, whereas the addition of alkalis accelerates it. Rona and Wilenko [1914] and Mauriac and Servantie [1922] found the optimal p_H to be approximately 8.

Several experiments were undertaken by the present writer in the following way. The mixed arterial and venous bloods of one or more rabbits were centrifuged and the corpuscles obtained in the usual way. They were suspended in an isotonic solution of 1/750 hydroxyquinoline sulphate and a weighed quantity of glucose was added. The suspension was then pipetted out into a number of flasks. One was kept as a control while to the others varying amounts of isotonic $N/2$ HCl or NaOH were added. About 3 cc. from each flask were then incubated and the glucose content estimated at intervals, while the rest of the suspension was centrifuged and the p_H of the

supernatant fluid determined electrometrically or colorimetrically. The writer is indebted to Dr M. Dixon for carrying out the p_H estimations. Figures typical of those obtained are shown in Table III and Fig. 1.

Table III.

Volume of reaction mixture cc.	cc. $N/2$ NaOH added	cc. $N/2$ HCl added	p_H	Glucose % degraded in 2 hours
10	—	0·3	7·39	0·002
10	—	—	7·73	0·026
10	0·25	—	8·10	0·061
10	0·50	—	8·60	0·068
20	1·50	—	9·38	0·021
20	2·00	—	9·70	0·003

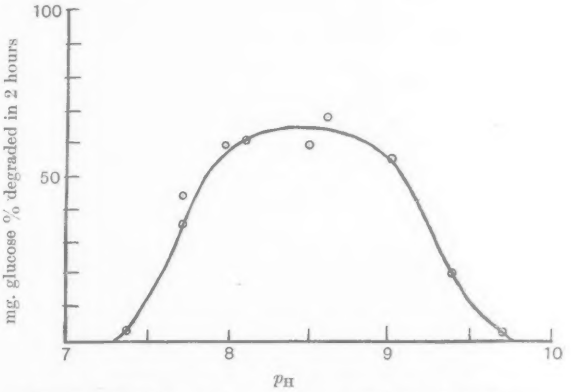

Fig. 1. The influence of p_H on the rate of glucose degradation.

The optimum p_H thus appears to lie between 8 and 9. One interesting fact which emerged from these estimations was the extreme constancy of the p_H of the corpuscular suspension before addition of acid or alkali. This was found to lie between 7·70 and 7·75. Although the addition of alkali caused a certain amount of haemolysis, this was not very marked until the p_H was in the neighbourhood of 9·5, since after centrifuging approximately the same volume of cells was obtained as was present before the addition of alkali. The addition of very small quantities of acid inhibited glycolysis very markedly.

Salts. Several experiments were carried out in which the corpuscles were suspended in isotonic solution of various ions. The following were tried:

$Na^{\cdot}$, $K^{\cdot}$, $Ca^{\cdot\cdot}$, $Mg^{\cdot\cdot}$, $Ba^{\cdot\cdot}$,

Cl', Br', I', F', NO_3', SO_4'', oxalate$''$, PO_4'''.

None of these was found to have any inhibitory action when in isosmotic concentration save fluoride and oxalate [cf. Macleod, 1913; Evans, 1922]; the former inhibited the reaction completely in small concentrations and glycolysis could not be restarted by either frequent washings or the addition of $CaCl_2$. The action of the fluoride ion in this and other instances is one of

great interest and is not at all understood. Oxalate inhibited in a similar way but to a less degree.

The degradation of other hexose sugars.

Portier [1903] and Griesbach and Oppenheimer [1913] showed that only those hexoses were degraded by blood corpuscles which were more or less closely allied to glucose. The present writer has tested fructose, mannose, galactose and rhamnose. The reducing power compared to glucose was found with pure solutions, and the necessary corrections have been made in all the figures quoted.

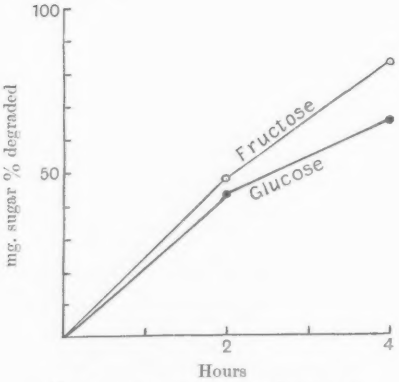

Fig. 2. The degradation of fructose by the adult rabbit's blood cells.

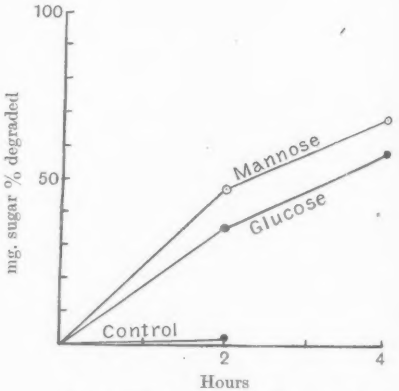

Fig. 3. The degradation of mannose by the adult rabbit's blood cells.

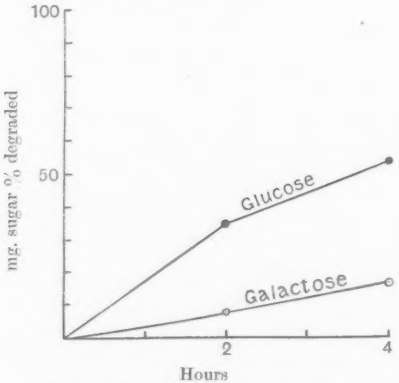

Fig. 4. The degradation of galactose by the adult rabbit's blood cells.

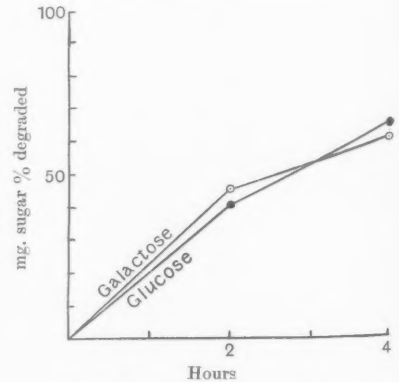

Fig. 5. The degradation of galactose by the infant rabbit's blood cells.

In the following experiments, the corpuscles were washed twice with saline and then divided into three portions: to one glucose was added, to one the sugar which was being tested was added, and the other was kept as

a control on the residual glycolysis. As a glance at Fig. 3 will show, the residual glycolysis was usually small, and in many cases was absent, so that the changes estimated occurred entirely in the added sugar. Figs. 2 and 3 show that fructose and mannose are degraded at about the same rate as glucose, the former usually slightly faster. Galactose (Fig. 4) is much more slowly attacked. Mixtures of glucose and fructose are degraded at the same rate as each alone, showing that the same mechanism is involved in the breakdown of each sugar. Rhamnose is not changed. These facts agree with those found for yeasts by Armstrong [1905] and Slator [1908], and in the main with those recorded by Herring, Irvine and Macleod [1924] in a study of the ability of various sugars to relieve insulin convulsions. These writers, however, found fructose to be relatively ineffective.

It is a matter of interest that the present writer has found that the corpuscles of young rabbits (aged about four weeks), in contrast to those of adults, were able to degrade glucose and galactose at equal rates (Fig. 5). Slator has stated that only those yeasts could ferment galactose which had become acclimatised to it, and perhaps the same occurs in this case.

From the evidence adduced here, there appears to be some fundamental connection between the stereochemistry of the sugar and the ability of the cell surface to degrade it. Until, however, we know more about the structure of the sugars, it will be impossible to postulate any satisfactory explanation.

SUMMARY.

1. The degradation of glucose by the corpuscle of the rabbit does not appear to involve the intervention of organic phosphorus compounds.

2. The optimum p_H is 8·5, but the optimal range is a wide one.

3. Glucose, fructose and mannose are degraded equally fast by the corpuscles of the adult rabbit, but galactose is much more slowly attacked. In the infant rabbit, however, galactose is degraded as fast as glucose.

The writer is indebted to Dr H. D. Kay for a specimen of calcium hexosediphosphate, and to Dr J. H. Quastel for a specimen of rhamnose.

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CLXVI. THE PREPARATION AND SOME PROPERTIES OF THE GLOBIN OF OXYHAEMOGLOBIN.

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I. Introduction.

It has long been known that haemoglobin consists of two portions, the iron-containing pigment, reduced haematin, combined with a protein, globin. The physical properties of the compound have been widely studied, and the protein obtained after removal of the pigment has been subjected to extensive chemical analysis. The globin, however, isolated by one or other of the modifications of the original method discovered by Schulz [1898] differs widely in solubility from the original haemoglobin. It is, rather, a metaprotein, similar to those obtained by the brief action of dilute acids on albumins or globulins. Thus, while soluble in either dilute acids or alkalis, it is insoluble at its isoelectric point, which according to Osato [1922] lies slightly on the alkaline side of the neutral point. Again, on treating its alkaline carbonate solution with a solution of haemin in alkaline carbonate and a reducing agent, the absorption spectrum of the solution is not that of haemoglobin. The spectrum is that of a haemochromogen and the substance is similar to other compounds of this class in that it is oxidised to haematin by the air. True globin under these conditions might be expected to yield haemoglobin and oxyhaemoglobin.

The protein to be described in this paper yields methaemoglobin on treatment with haemin dissolved in alkaline carbonate. Typical haemoglobin and oxyhaemoglobin can thence be obtained. A survey of the literature reveals scattered claims to have resynthesised oxyhaemoglobin in spectroscopically observable amounts from the products of its partial disruption. Two such claims are those of Bertin-Sans and de Moitessier [1892] and of Anson and Mirsky [1925; cf. Barcroft, 1925]. The evidence on which these claims are based will be discussed later, but it seems doubtful if any of these workers prepared true globin in sufficient quantities to admit of extensive investigations, or reasonably free from the products of its own denaturation.

The experiments described in this paper fall into three main groups. A method is given for preparing globin in quantities sufficient for many experiments. A preliminary account of some of the properties of this protein is given. Details are given of a method of preparation of protoporphyrin [Fischer and Putzer, 1926]—the porphyrin of haemoglobin—which seems more

practicable than the methods described in the literature. The other experiments deal with the reactions of globin with haemin, porphyrins and some metallic derivatives.

II. The preparation of globin.

Anson and Mirsky's [1925] view, that acid haematin obtained directly from oxyhaemoglobin by the action of dilute acid is a compound of globin and haematin, has been shown by Keilin [1926] to be unnecessary for the explanation of its properties. The apparent solubility of the pigment is due to the protective action of the protein. In the Schulz method of preparing denatured globin this protective action is inhibited by the use of alcohol which simultaneously denatures the globin. Various attempts have been made to remove the haematin from such a solution without the use of alcohol, by great dilution and the use of charcoal or other adsorbing agents. These methods are, however, quite unsuitable for the preparation of any reasonable quantity of globin.

It seemed probable that the best conditions would be obtained by the use of the minimum amount of acid necessary to decompose all the oxyhaemoglobin at the lowest temperature at which ice was not deposited. After a few trials it was observed that the stroma proteins, particularly when swollen with ether, have a great power of absorbing haematin from an acid solution of haematin protected by globin. The addition of carefully purified kieselguhr rendered filtration easy. The following technique, while it has been modified to permit of the preparation of larger quantities of globin, yields a solution suitable for spectroscopic experiments. A scrupulous attention to detail is essential if a reasonable yield of about 40 %, calculated on the amount of oxyhaemoglobin originally present, is to be obtained. Higher yields have been obtained; variations in samples of blood appreciably affect the yield. All the apparatus must be boiled in chromic acid before each experiment, and it is advisable to use it solely for the purpose of preparing globin. The centrifuge must be kept carefully cleaned and it was found advisable to keep one instrument for the sole purpose of centrifuging the blood from which the globin was to be prepared. The air of the room must be free from any traces of volatile organic substances or acids. The most suitable room is a chamber in a refrigerating plant. If the work is carried out in an ordinary laboratory a cool day is absolutely essential and the preparation should be conducted in a small room in which no other chemical operations are being carried out. Most of the work has been done with ox blood but the method has been found to hold good for the blood of other mammals.

The defibrinated blood was centrifuged as rapidly as possible and the corpuscles washed four times with 0·9 % sodium chloride solution. An equal volume of water was added to the mass of corpuscles and the solution of oxyhaemoglobin so obtained could be stored for three or four days at 0° when so desired. In a bath at $-2°$ were placed the following: a test-tube containing

a few cc. of 2*N* hydrochloric acid and a 1 cc. pipette graduated in hundredths of a cc.; a test-tube with rods standing in distilled water; several test-tubes each containing 5 cc. of the cooled oxyhaemoglobin solution; a dry 70 cc. flask with a rubber stopper; a flask containing three times as much distilled water as the total volume of the oxyhaemoglobin solution; another flask containing some ether which had been well washed with water to remove alcohol and peroxides. In an ice-chest were a measuring cylinder and some small conical flasks and in each a funnel with a fluted filter-paper. A Büchner funnel in an ice-chest was used for dealing with larger quantities of globin. All the apparatus and solutions were thoroughly cooled before the experiment was started.

To 5 cc. of the cooled oxyhaemoglobin solution was added enough 2*N* hydrochloric acid to convert it completely to acid haematin in three or four minutes; about 0·68 cc. was required. The right amount was found by trial, excess being avoided. Immediately on addition of the acid the mixture was stirred with a cooled rod and a hand spectroscope was used to see that no oxyhaemoglobin remained. 15 cc. of the ice-cold water were placed in the cooled cylinder and 5 cc. added to the viscid mass of acid haematin. On stirring it dissolved and was poured into the 70 cc. flask, the test-tube being washed out with the remaining 10 cc. of water into the same flask. About 7 cc. of the ether and 1 g. of purified kieselguhr were added. The flask was closed with the stopper, removed from the bath, wrapped in a cloth and shaken vigorously for thirty seconds, cooled and shaken again for thirty seconds. After standing in the bath for a minute it was filtered in the ice-chest.

During these operations every care must be taken to prevent the temperature rising above 0°. It has been found that under favourable conditions the amount of denaturation due to the ether is small. Most of the loss occurs during the decomposition of the oxyhaemoglobin. The diluted solution of acid haematin has been kept over night at 0° without much further denaturation occurring. After filtration *N*/2 ammonia was immediately added until the maximum precipitate was obtained and the metaprotein filtered off in the ice-chest. The solution was placed in an open dish in a vacuum desiccator and the ether removed by a vacuum pump. After removal of the ether the globin solution is stable at room temperature. After dialysing away the salts at 0° a sample was dried at 0° over sulphuric acid in the vacuum of a mercury pump. It remained undenatured in the solid state at room temperature for several weeks.

The ox globin thus obtained contained up to about 2 % of methaemoglobin. It was freely soluble in distilled water over the range p_H 5–10. It is easily denatured by acids, strong alkalis or organic solvents. It is not precipitated by saturating its solution with sodium chloride but is partially precipitated by half-saturation with ammonium sulphate and completely by 60 % saturation. If this is carried out at 0° most of the globin can be recovered undenatured from the precipitate.

This globin reacts with a solution of haemin in sodium carbonate to give methaemoglobin, thus realising the scheme proposed by Küster [1910]. In 1 % sodium carbonate solution it does not yield a haemochromogen with haematin and ferrous sodium tartrate. It appears to combine with porphyrins, producing a change in the spectrum of a solution of haematoporphyrin in alkaline carbonate which can be readily observed with a direct vision spectroscope. Globin is rapidly denatured in solution by sunlight in the presence of small amounts of haematoporphyrin. Such a solution after exposure to light is incapable of yielding methaemoglobin on treatment with haematin.

III. The reaction between globin and haematin.

In addition to ordinary haemin, the iron derivatives of haematoporphyrin and mesoporphyrin were used. A solution of any one of the three in dilute sodium carbonate reacted with globin to give the corresponding methaemoglobin. On reduction with alkaline ferrous tartrate or ammonium sulphide the corresponding haemoglobin was obtained, which, on shaking with air, formed the corresponding oxyhaemoglobin. The position of the α band of the oxyhaemoglobin obtained from haemin was within 5 Å.U. of that of the oxyhaemoglobin from which the globin was prepared. In the absence of any reducing agent these oxyhaemoglobins yield up their oxygen to vacuum and reabsorb it from the air. Carbon monoxide, in the absence of a reducing agent, displaces the oxygen forming the corresponding CO-haemoglobin. Potassium ferricyanide oxidises each to the corresponding methaemoglobin.

The best indication of the purity of a solution of oxyhaemoglobin is its colour. Artificial oxyhaemoglobin prepared from a solution of haemin in carbonate is apt to be slightly brownish. This was found to be due, not to the globin, but to the fact that in alkaline solution haematin rapidly suffers slight decomposition. If haemin crystals be added to a faintly alkaline solution of globin, avoiding an excess of pigment, the oxyhaemoglobin thence obtained has a colour similar to that of diluted blood. For the conversion of methaemoglobin to oxyhaemoglobin on the alkaline side of p_{H} 7 care is necessary in the selection of a suitable reducing agent. Sodium hydrosulphite is the chief reducer in common use which is free from nitrogenous substances capable of yielding haemochromogens. In its presence an alkaline solution of haemoglobin does not yield oxyhaemoglobin on shaking with air. After several trials it was decided to use throughout a solution of sodium ferrous tartrate in sodium carbonate, being a nitrogen-free modification of the reagent of Stokes. Fig. 1 and Table I show the positions of bands of derivatives of the artificial haemoglobins as measured by the Hartridge reversion spectroscope. It has been frequently found that the bands of the artificial oxyhaemoglobin may lie up to four Ångstrom units to the violet of those of the original oxyhaemoglobin. It has not yet been found possible to determine whether any significance is to be attached to this fact.

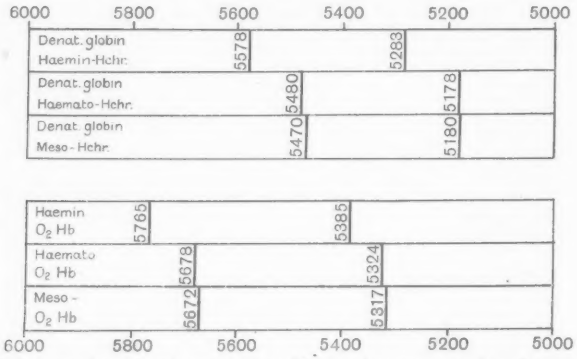

Fig. 1. Influence of porphryrin on spectra of haemochromogens and oxyhaemoglobins.

Table I. *Wave-lengths of bands of derivatives of different porphyrins in Ångstrom units.*

	Diluted ox blood for comparison			Haemin (Protoporphyrin)			
Porphyrin-globin	—	—	—	6224	5673	5426	5079
Methaemoglobin	6046	5766	5390	6037	5758		5395
Oxyhaemoglobin		5768	5387		5765		5385
CO-haemoglobin		5699	5357		5693		5352
Denat. globin haemochromogen		5578	5283		5578		5281

	Haematoporphyrin				Mesoporphyrin			
Porphyrin-globin	6126	5590	5348	5001	6120	5586	5341	4992
Methaemoglobin	5980	5682		5341	5960	5662		5341
Oxyhaemoglobin		5678		5324		5672		5317
CO-haemoglobin		5623		5286		5603		5271
Denat. globin haemochromogen		5480		5178		5470		5180

The combination of globin with haematin to form methaemoglobin can occur over a wide range of hydrogen ion concentration. On the acid side of p_H 5 combination is less than 50 %. On the alkaline side the limit has not been ascertained, combination being unaffected by p_H 10.

Fig. 1 shows that the differences in constitution between the three haematins are not sufficient to render any one of them incapable of reacting with globin. The strong tendency to combine with haematin possessed by globin is well shown by the fact that a suspension of the insoluble calcium salt of haematin in a faintly alkaline solution of globin slowly forms methaemoglobin on keeping at 0°.

IV. The equilibrium between methaemoglobin and acid haematin.

With a view to obtaining the best conditions for the preparation of globin some preliminary studies were carried out on the above equilibrium. An acid solution of globin, denatured globin, and haematin was prepared at 0° as described in the preparation of globin. Varying amounts of 0·2*N* ammonia were added to 10 cc. samples and the haematin removed at 0° with ether and kieselguhr. On the filtrates two determinations were performed.

(1) A colorimetric estimation of the proportion of haematin still remaining.

(2) The amount of undenatured globin (both free and combined as methaemoglobin), estimated as oxyhaemoglobin by adding excess of haemin in alkali, reducing and reoxidising.

The results are shown in Fig. 2.

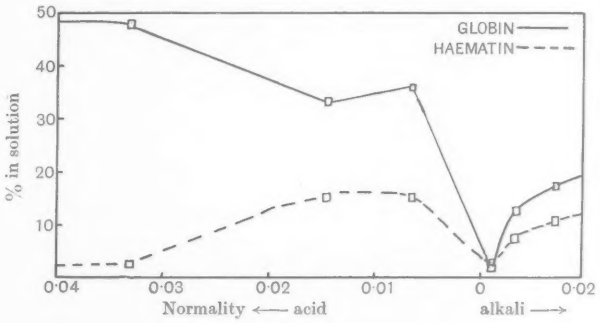

Fig. 2.

The haematin values are somewhat high owing to the inclusion of small amounts of other pigments. A certain amount of recombination to methaemoglobin occurs when the solution is definitely acid. It is noticeable at p_H 4. Although at p_H 5 methaemoglobin begins to dissociate into haematin and globin, the degree of dissociation only gradually increases with increase of hydrogen ion concentration and is substantially complete between p_H 3 and 4. Some experiments on the decomposition of oxyhaemoglobin at 0° in buffer solutions showed that decomposition was complete in about three minutes at p_H 1·9, while at p_H 2·3 ten minutes were required. Near the neutral point practically all the globin, denatured globin and pigment are precipitated together as a bright red mass of "parahaematin" [Keilin, 1926]. On the alkaline side both haematin and globin appear in the filtrate as alkaline methaemoglobin. Over the range covered by these experiments a certain proportion of the globin is still uncombined owing to the removal of some of the haematin by the treatment with ether.

V. The preparation of protoporphyrin.

1900 cc. of fresh defibrinated sheep's blood were inoculated with a culture of *B. coli*, and allowed to stand three days at 20°. To the completely reduced blood thus obtained 880 cc. concentrated hydrochloric acid were added from a separating funnel fitted with a glass jet introduced beneath the surface of the reduced blood. The bottle was thus filled to the top of the neck and a greased stopper was inserted to exclude all air. It was violently shaken and after two hours the contents were squeezed in strong linen cloth. The solid

residue contained most of the porphyrin as its sparingly soluble hydrochloride. The small amount of porphyrin in the expressed acid was neglected. 1·5 l. of 97 % alcohol were used for extracting the porphyrin hydrochloride from the precipitated proteins and the liquid was filtered. The solution (1450 cc.) containing the porphyrin hydrochloride was poured with stirring into a mixture of 700 cc. 20 % sodium hydroxide solution and 30 cc. saturated sodium tartrate solution. The temperature rose to 40° and the sodium salt of the porphyrin separated as a fine micro-crystalline precipitate. After standing over night the supernatant fluid was decanted. The precipitate was filtered off and washed thoroughly with 50 % alcohol in which the sodium salt is practically insoluble. The porphryin thus obtained formed a nearly black mass with a conchoidal fracture, and, after drying over calcium chloride in a vacuum, weighed 3·5 g. The preparation was somewhat hygroscopic and readily dissolved in distilled water giving an opalescent solution. It was immediately precipitated by the addition of alkali, salts or dilute acids. In 30 % sulphuric acid a fluorescent purple-red solution was formed, greenish in thin layers. The porphyrin is only sparingly soluble in hydrochloric acid. From its solutions in acids it is precipitated on dilution with water.

The mesoporphyrin hydrochloride was prepared from haemin by heating with formic acid and colloidal palladium as described by Fischer and Putzer [1926].

Haematoporphyrin hydrochloride was prepared from haemin by a slight modification of Nencki's method as described by Hill [1925].

The haemin was prepared by a method similar to that of Schalpejew [1885] employing, however, corpuscles laked with a small amount of ether in place of whole blood, in order to economise in acetic acid.

The chemical relationships between these porphyrins have been elucidated by Fischer and Müller [1925].

VI. The reaction of globin with porphyrins.

When a solution of haematoporphyrin or mesoporphyrin in dilute sodium carbonate is added to a solution of globin the colour of the solution undergoes an immediate change from orange to pink. The absorption bands, previously ill-defined, become exceedingly sharp and intense (see Fig. 3 and Table I). The porphyrin is no longer removed by adding calcium carbonate and filtering. Denatured globin does not show these effects. It seems probable that a definite compound of porphyrin and globin is formed. Attempts to introduce iron directly into this compound without denaturing the globin were unsuccessful.

By using a series of buffer solutions and a neutral solution of haematoporphyrin protected by gum arabic it was found that this reaction did not occur in solutions more acid than p_H 5.

On adding haemin in sodium carbonate to a solution of haematoporphyrin-globin methaemoglobin is formed. The ordinary spectrum of porphyrin in

alkali can also be seen, indicating a displacement of the porphyrin by the haematin.

In the case of protoporphyrin the reaction between its sodium salt and globin is exceedingly slow, and several weeks would be necessary in order to reach a state of equilibrium. It can be expedited by preparing a solution of protoporphyrin in 30 % sulphuric acid with the addition of denatured globin as a protective colloid with which it does not combine. If such a solution be made alkaline with sodium carbonate and immediately poured into a

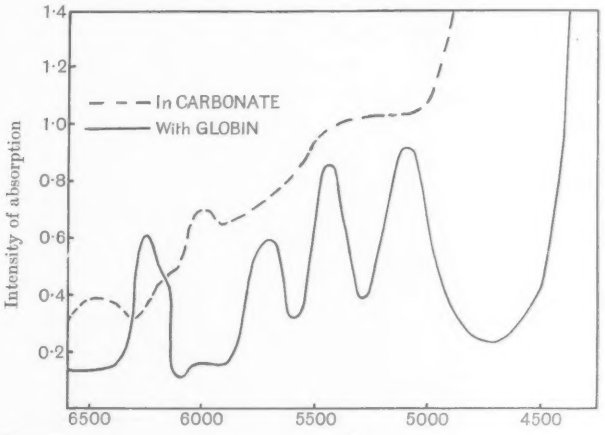

Fig. 3.

solution of globin combination occurs. Ammonia cannot be used instead of sodium carbonate. If to the globin solution a considerable excess of sodium carbonate be added the acid solution of protoporphyrin may be added directly to the globin without denaturation occurring. The colour of such a solution is at first orange and after four or five seconds turns pink.

With the help of a spectrophotometer a quantitative study of this reaction was carried out. Various amounts of protoporphyrin were added to equal quantities of the alkaline globin solution, and the difference between the intensity of absorption at 6220 Å.U. and at 6400 Å.U. was measured in each case. The same globin solution was titrated against haemin by a similar means, the difference in absorption between the α band of oxyhaemoglobin (5780 Å.U.) and the band of alkaline haematin (6200 Å.U.) being measured. It was to be expected that each of these curves would show a discontinuity when all the globin had entered into combination, provided that both substances were undissociated. Within the limits of experimental error it was found that in the protoporphyrin-globin compound each molecule of globin combines with

the same number of molecules of porphyrin as it does with haematin in the form of oxyhaemoglobin.

The details of the method are as follows:

0·0314 g. of the sodium salt of protoporphyrin was dissolved in a mixture of 5 cc. water, 1·5 cc. pure sulphuric acid and 9 cc. glacial acetic acid. When completely dissolved the solution was made up with water to 25 cc. This mixture of acids was found by trial to be the minimum concentration of total acid that would readily dissolve the porphyrin.

To 2 cc. samples of globin solution, of approximately 0·9 % as determined by titration with haemin, were added 14·5 cc. water and 6·5 cc. of 15 % sodium carbonate solution. Varying amounts of protoporphyrin solution were dropped in from a micro-burette with brisk stirring. After addition the solution was made up with distilled water to 50 cc. The intensity of absorption was immediately measured in a 1 dm. tube.

For the titration with haemin, 0·0337 g. of crystalline haemin was dissolved in a little water containing 1 cc. of 15 % sodium carbonate solution and made up to 250 cc.

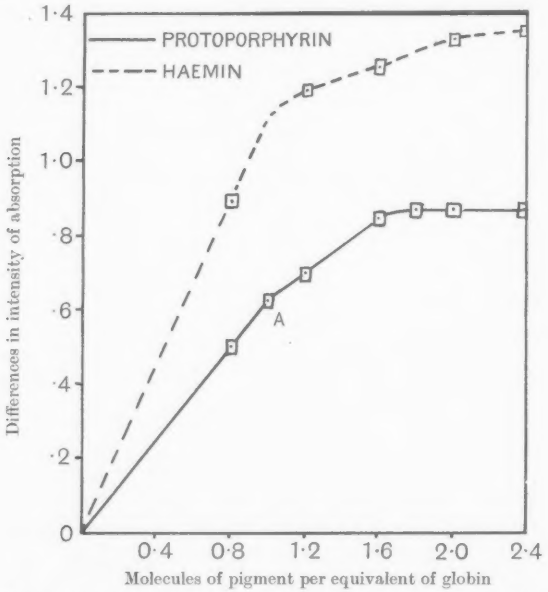

Fig. 4. At the point A the slope of the curve decreases by one half.

To 1 cc. samples of the globin solution were added varying amounts of haematin from a micro-burette and a constant sufficient amount of alkaline ferrous tartrate solution, great excess of the latter being avoided. After reduction the solution was gently aerated to oxidise both the haemoglobin

and the remaining reducing agent. It was made up to 50 cc. and immediately read in a 1 dm. tube. From the accompanying figure, Fig. 4, the experimental evidence appears to favour the conclusion stated above.

VII. The reaction of globin with metallo-porphyrins.

Mesoporphyrin was chosen as the porphyrin most suitable for the demonstration of this characteristic of globin, owing to the ease with which its metallic derivatives can be isolated in a crystalline condition. The method of their preparation was based on that described by Fischer and Putzer [1926] for the metallic derivatives of protoporphyrin. After washing with 10 % acetic acid and with water, the metallic derivative was suspended in 1 % sodium carbonate solution and its absorption spectrum measured in the presence and in the absence of globin. Before measurement the solutions containing globin were shaken with a little calcium carbonate and filtered. This removed any pigment not in combination with the globin. The time necessary for combination apparently depended on the degree of solubility of the pigment in the dilute sodium carbonate. The tin derivative combined at once; the copper incompletely even after 12 hours. No other colloidal substance could be found, *e.g.* denatured globin, serum protein, capable of producing a similar effect. It can however be simulated by the addition to the pigment of excess of a suitable organic solvent, *e.g.* pyridine. In the latter case the absorption bands, though sharp compared with those of the suspension, differ in wavelength from those of the globin compound. The quality of the change in the absorption spectrum is very similar to that observed in the case of the free porphyrins.

The metallo-porphyrins of manganese and cobalt [Laidlaw, 1904] can be reduced to a form reoxidisable by the air. Their complete reduction occurs only under conditions which denature globin. Consequently the attempt which was made to obtain information concerning the effect of globin on their oxidation failed. Metallic derivatives of haemato- and proto-porphyrin have been observed to yield similar globin compounds. We have been unable to observe this effect with uroporphyrin or its iron and copper derivatives. Dr Allott kindly supplied a specimen of this porphyrin. The zinc derivatives of haemato- and of meso-porphyrin, when combined with globin, and in the absence of any removable pigment, possess a spectrum in which the α band has two well-defined maxima. In ether the α band of the free zinc porphyrin, though sharp, appears single.

The changes in the absorption spectra of some metallic derivatives of mesoporphyrin as the result of combination with globin are shown in Table II. Denatured globin can take up in solution some metallo-porphyrins, but the appearance and position of the bands are different from those of the metallo-porphyrin-globin compounds.

Table II. *The effect of globin on the spectra of metallic derivatives of mesoporphyrin.*

Metal	Medium	Wave-length of bands, Å.U. α	Wave-length of bands, Å.U. β	State of pigment without globin	Appearance of bands
Mn	Carbonate	5924	5537	Solution	Indefinite
	Globin	5803	5469		More definite
Co	Carbonate	5600	5260	Suspension	Indefinite
	Globin	5587	5252		Sharp
Ni	Carbonate	5627	5261	Suspension	Indefinite
	Globin	5490	5119		Very sharp
Cu	Carbonate	5779	5409	Suspension	Indefinite
	Globin	5577	5215		Very sharp
Zn	Carbonate	5735	5362	Suspension	Indefinite
	Globin	{5837 / 5724}	5418		Sharp
Sn	Carbonate	5714	5356	Solution	Sharp
	Globin	5740	5338		Very sharp

Discussion.

Any consideration of the various claims existing in the literature to have resynthesised oxyhaemoglobin from a pigment and a protein involve an enquiry into the value to be attached to spectroscopic evidence, with regard to the particular experimental conditions under which it is obtained. In dealing with the absorption spectra of solutions containing more than one light-absorbing substance, not only the position but the relative intensity of the bands must be borne in mind. An experiment [cf. Keilin, 1925] will illustrate the necessity for the utmost reserve in interpreting such evidence. If in a test-tube 2 cc. of ox blood that has been diluted with 100 volumes of water be treated with 0·5 cc. of 10 % sodium hydroxide the protein is completely denatured in a few minutes. 2 cc. of the same diluted blood are then added. If the solution be reduced with ferrous tartrate and sodium carbonate, without the addition of any more sodium hydroxide, the spectrum of the reduced haemoglobin is practically invisible and on reoxidation the spectroscopic evidence might easily lead to the conclusion that haemochromogen had been oxidised to oxyhaemoglobin. If, instead of oxidising the solution, the hydroxyl ion concentration be gradually decreased, the haemochromogen band becomes less intense and shifts up to 10 Å.U. towards the red. The band of reduced haemoglobin gradually becomes more noticeable. Parahaematin, described by Keilin [1926], has a red colour and its absorption spectrum bears some resemblance to that of oxyhaemoglobin. The bands however are ill-defined and lie somewhat to the violet of the corresponding bands of oxyhaemoglobin. The possibility of parahaematin formation near the neutral point, and the low selectivity of the absorption of both haematin and methaemoglobin as compared with oxyhaemoglobin, may have led some workers to assume the resynthesis of oxyhaemoglobin on an insufficient experimental basis.

Bertin-Sans and de Moitessier [1892] claimed to have separated from oxyhaemoglobin a globin from which the original oxyhaemoglobin could be synthesised by neutralising very slowly an alkaline solution containing this globin together with added haematin. The ox blood was coagulated with ether and treated with a boiling solution of 10 % tartaric acid in ethyl alcohol. They record no measurements of the position of the bands of the resynthesised oxyhaemoglobin. It is difficult to see how any could have been detected in the presence of the relatively very large amounts of parahaematin which must have been formed by their procedure. It is very difficult to remove the last traces of ammonium sulphide from solutions; and since they used this reducing agent their synthetic CO-haemoglobin might have been CO-haemochromogen, owing its formation to the denatured globin present and a small amount of ammonium sulphide which had escaped oxidation. Again no data of the position of the bands are recorded and the spectra are of a similar pattern and the corresponding bands differ very slightly in wave length. The spectrum of a mixture of parahaematin and haematin simulates that of alkaline methaemoglobin.

If denatured globin be precipitated between p_H 6 and 7 by salts or alcohol, instead of at its isoelectric point, p_H 8, it will redissolve in distilled water after washing. It is reprecipitated at p_H 8. This probably explains the solubility in distilled water of the protein prepared by these workers. Such a neutral solution of denatured globin was prepared during the course of the experiments described in this paper and tested for freedom from (undenatured) globin. On treatment with a nearly neutral solution of haemin in alkali it yielded a clear red solution of denatured globin parahaematin. Neutralisation and subsequent oxidation of a solution of denatured globin haemochromogen convert it into parahaematin. No experiment could be found recorded in which, in any solution of globin, the methaemoglobin present was estimated by reduction and reoxidation without the addition of haematin, and the increased yield of oxyhaemoglobin due to the addition of haemin in alkaline carbonate to this solution determined. This test is illustrated in Fig. 4. Unless the greatest precautions are observed denatured globin prepared by the Schulz method may well contain, as does the globin prepared by the method of this paper, traces of methaemoglobin. This essential precaution was unfortunately neglected by even so careful a worker as Menzies [1894]. Perhaps it is significant that, with the exception of Menzies, all these workers have claimed to obtain a resynthesis of oxyhaemoglobin only under the conditions essential to the formation of parahaematin.

The globin prepared as described in this paper may be distinguished from that prepared by any modification of Schulz's process by the following tests. It is soluble over the range p_H 5–10. It is precipitated from a neutral solution by 60 % saturation with ammonium sulphate and provided that the precipitation is carried out at a low temperature the precipitate is readily soluble in solutions containing less than this amount of the salt. In a pure state it

does not yield a haemochromogen in a solution made alkaline with sodium carbonate. Under these conditions denatured globin readily yields its haemochromogen.

Experiments dealing with the conversion of haemochromogen to haemoglobin by decrease in the hydroxyl ion concentration of the solution do not appear to have excluded the possibility that in the solutions two proteins were present simultaneously. It was found that globin could remove the pigment from denatured globin haemochromogen and so produce haemoglobin. In a solution containing globin, denatured globin and haematin, the pigment may well combine with either protein according to the degree of alkalinity.

In the preparation of globin, the action of the acid on oxyhaemoglobin always denatures a certain proportion of the protein. This denatured globin can be readily freed from globin. It is then insoluble at its isoelectric point. Its alkaline solution yields on treatment with haemin in sodium carbonate solution haemochromogen but no haemoglobin. Its neutral solution as mentioned above yields parahaematin and no methaemoglobin. If denaturation of globin be defined either in terms of the alteration in solubility at the isoelectric point or in terms of its reaction with haemin in alkaline carbonate, the fact of its preparation from oxyhaemoglobin shows that separation of the pigment does not necessarily involve denaturation.

The combination of globin with compounds not containing iron, *i.e.* porphyrins and their other metallic derivatives, indicates that the porphyrin residue plays a part in the formation of haemoglobin independently of the iron. The following evidence may suggest an explanation of the mechanism of the action of globin on such compounds. The spectrum of a solution of a porphyrin in the presence of globin is similar in the sharpness of the bands to that of the same porphyrin in true solution in an organic solvent [cf. Keilin, 1926]. Adair [1925] has shown that each molecule of globin is combined with four molecules of pigment in oxyhaemoglobin. Hartridge and Roughton [1923] have shown that the reduction of oxyhaemoglobin is a monomolecular reaction. It would therefore seem probable that in a molecule of any globin-pigment compound the pigment molecules are separated from one another by distances large compared with their own molecular dimensions. The quantitative relationships between protoporphyrin globin and oxyhaemoglobin are in agreement with this conception. Haematin displaces haematoporphyrin rapidly and protoporphyrin slowly from their globin compounds. These facts taken in conjunction may suggest that protoporphyrin globin is haemoglobin without iron.

SUMMARY.

1. From the oxyhaemoglobin of ox blood has been prepared a protein which is considered to be the true globin of this conjugated protein.

2. This globin is soluble in the presence or absence of salts over the range p_H 5–10.

3. Spectroscopic evidence is presented of the identity of the reconstituted oxyhaemoglobin and the original oxyhaemoglobin from which the globin of the former was prepared.

4. Globin reacts with haematin over the range p_H 5–10 to give methaemoglobin. This equilibrium has been investigated on the acid side.

5. Evidence is given of the occurrence of compounds of globin with certain porphyrins and their metallic derivatives.

6. Oxyhaemoglobins have been prepared from the iron compounds of haematoporphyrin and mesoporphyrin.

The authors desire to record their gratitude to Sir F. G. Hopkins for his continual interest and encouragement, to the Government Grant Committee of the Royal Society for a grant covering the cost of certain apparatus, and to the Cambridge University Medical Grant Committee for the gift of a spectrophotometer.

One of them (R. H.) is indebted to the Medical Research Council for a grant.

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CLXVII. NOTE ON THE ANTI-RACHITIC ACTION OF IRRADIATED SAWDUST.

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(*Received October 27th, 1926.*)

No adequate experimental explanation has as yet been offered in interpretation of the results recorded by Hume and Smith [1923] in which increased growth was observed when rats on a diet deficient in fat-soluble vitamins inhabited glass jars previously irradiated. The suggestion that the effect might be due to the ionisation of the irradiated air was soon disproved [Webster and Hill, 1924; Hume and Smith, 1924; Nelson and Steenbock, 1925; Hughes, Nitcher and Titus, 1925] and it was shown to be connected with irradiation of the sawdust, used as bedding in the original experiments. In their last communication on this subject, Hume and Smith [1926], whilst eliminating the possibility of any kind of secondary radiations from the sawdust having an anti-rachitic effect, find their other results "not so easy of interpretation." In these experiments, the rats were kept in glass jars, separated from the irradiated sawdust by a wire grid. The authors state, however, that "the only conclusion for which the evidence is satisfactory is that irradiated sawdust exercises a beneficial action on growth and calcification when consumed by the rats."

The fact discovered independently by Hess, Weinstock and Helman [1925], Steenbock and Black [1925] and by ourselves [1925] that sterols acquire anti-rachitic properties on irradiation, suggested to us that the effect of irradiated sawdust on growth might be due to the presence in it of sterols. The occurrence of these substances in all vegetable tissues is well known, and crystallised phytosterols have been isolated from wood, bark, cork and resins, all of which may form the constituents of mixed sawdust.

We tested this suggestion experimentally by extracting with chloroform 250 g. of mixed sawdust, as used for bedding in our animal experiments, until Liebermann's sterol reaction was no longer given by the extract. The chloroform extract was concentrated *in vacuo* and left a yellowish resin (3·2 %), possessing the characteristic sawdust odour. No attempt was made to isolate the sterol (or terpene?) in a pure condition, since the purpose of the experiment was to test the anti-rachitic action of the whole extract. The resin was irradiated for half an hour (40 cm. distant from a Cooper-Hewitt mercury-vapour lamp) and administered, dissolved in olive oil, to rats kept on Sherman-Pappen-

heimer's diet No. 84. The control rats developed typical rickets, whilst the animals receiving 5 mg. *per diem* of irradiated extract of sawdust were entirely protected, as proved by radiographic and chemical evidence.

The extracted sawdust no longer acquired, when irradiated, the power of fogging a photographic plate, but the resinous extract showed this property even when exposed for a short time only to ordinary white light. The action on a photographic plate of cod-liver oil, irradiated vegetable oils and sterols has led many recent writers to assume a connection between anti-rachitic and photochemical activity (see especially Vollmer and Serebrijski [1926]). The experimental results obtained by Kugelmass and McQuarrie [1924] in particular, were adduced in explanation of the anti-rachitic action of irradiated sawdust, although these authors [1925] soon withdrew their original claims. Their results were explained by themselves and others as being due, not to radiations, but to volatile "peroxides" (the "Russell effect"; for a review of the literature see Niederhoff [1926]). The latter substances are, however, not produced when sterols are irradiated *in vacuo* or in hydrogen and the effect on the photographic plate is absent [Hamano, 1925]. On the other hand, the anti-rachitic activity of cholesterol, when irradiated under similar conditions (nitrogen atmosphere), is not only more pronounced but also more stable than that of air-irradiated cholesterol [Rosenheim and Webster, 1926]. This evidence together with other facts which we have obtained appears to be definitely against any association between anti-rachitic activity and the chemical action on a photographic plate of volatile "peroxides."

In explanation of the growth effect observed by Hume and Smith in their original experiments, we may now exclude the effect of (1) ionisation of irradiated air, (2) secondary radiations, and (3) volatile "peroxides." It seems justifiable to conclude, from the results of the above-described experiment, that the increased growth under the experimental conditions of Hume and Smith is due to the ingestion by the rats of sawdust, the sterols of which had become anti-rachitic by irradiation.

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CLXVIII. THE NATURE OF FEARON'S COLOUR REACTION AND ITS NON-SPECIFICITY FOR VITAMIN A.

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Fearon [1925] described a colour reaction which he considered to be associated with vitamin A and suitable for colorimetric work. Attracted by the permanence of the reaction, Willimott and Moore [1926], as well as Euler, Myrbäck and Karlsson [1926], have attempted to make it the basis of a method for the quantitative colorimetric estimation of vitamin A. The reaction consists essentially in the production of a stable bright rose colour by the addition of trichloroacetic acid to a mixture of cod-liver oil with a phenol (pyrogallol). The pigment formation is assumed to be due to the condensation of an aldehydic chromogenic constituent of the oil (= vitamin A) with the phenol, and is intensified by the addition of an oxidising agent (benzoyl peroxide).

It is surprising that neither the discoverer of the reaction nor his followers have applied the test to the unsaponifiable fraction of cod-liver oil and thereby obtained more definite evidence with regard to its specificity for vitamin A. It is now well established that this fraction contains the whole of the growth-promoting material present in the oil and that the soaps are free from vitamin A. Consequently the first condition to be satisfied by any such test must be a positive reaction when applied to the unsaponifiable fraction of an oil [Rosenheim and Drummond, 1925]. Since we found, when carrying out this crucial test [Rosenheim and Webster, 1926], that Fearon's colour reaction is not given by the unsaponifiable fraction of cod-liver oil, it is evident that this reaction has no relation to vitamin A, for the estimation of which it was suggested. Further, apart from the non-specificity of the reaction, it was found that trichloroacetic acid, intended to act as a condensing agent, itself gave rise to a reaction of an entirely different character and colour, in the absence of a phenol. Since the relative amounts of the two chromogens entering into the reaction vary in different oils, the resulting mixture of colours cannot be estimated by the ordinary colorimetric methods.

Experimental.

5 g. cod-liver oil, giving a strong Fearon's reaction, were saponified with alcoholic potash. The unsaponifiable portion, prepared in the usual way, gave an intense arsenious chloride reaction but was entirely negative to Fearon's

test. The same negative result was obtained with different liver oils, and also in the case of sardine oil, a fish body oil (see below). A preparation of the unsaponifiable fraction of cod-liver oil, freed from cholesterol by means of digitonin and kindly supplied to us by Prof. Drummond, gave identical results.

The nature of the chromogen. The substance responsible for Fearon's reaction is not destroyed by saponification, but can be recovered from the soap solution. After acidifying the vitamin-free soap solution, the fatty acids were extracted with pure ether and were found to give the reaction[1]. The mixed fatty acids were separated by the well-known lead acetate process into two fractions. The unsaturated fatty acids liberated from the ether-soluble lead salts contained the chromogen, whilst the saturated fatty acids from the ether-insoluble lead salts gave a negative result. The chromogen is therefore associated with the unsaturated fatty acids. It can further be shown that it occurs in the original oil as an ester in combination either with glycerol or another alcohol (cholesterol?). This follows from the fact that the chromogen cannot be removed from the oil by shaking with 2 % KOH at room temperature and is only converted into a water-soluble salt by boiling with alcoholic potash.

We would suggest, as a working hypothesis, that the chromogen is an aldehydic oxidation product of a highly unsaturated fatty acid of the type of clupanodonic acid, $C_{22}H_{34}O_2$. This acid was first isolated by Tsujimoto from Japanese sardine oil (from *Clupanodon melanosticta*) and subsequently also from cod-liver oil, other fish oils and from green algae [Tsujimoto, 1922, 1925]. This suggestion is in agreement with the fact that all vegetable oils are negative in Fearon's test. In these oils clupanodonic acid cannot be detected by the "bromide" test, whilst we were able to isolate as much as 20 % of the decabromide of clupanodonic acid, $C_{22}H_{34}O_2Br_{10}$, from the fatty acids of an animal oil which gave an intense Fearon's reaction. It may further be pointed out that acids of similar type are easily oxidised to aldehydic fatty acids, which could act, owing to their aldehydic functions, as Fearon's postulated chromogen when condensed with phenols.

Comparative colour- and feeding-tests. When testing a large number of cod-liver oils and other liver oils, dissolved in light petroleum according to Fearon's directions, we found that only relatively few of them gave the bright rose colour, which was considered by him as characteristic for vitamin A. A large number gave a pea-green, others an intense emerald green and still others an equally intense greenish-blue colour. After addition of benzoyl peroxide the colours changed into carmine, reddish purple and various shades of magenta respectively. Similar erratic changes were recorded by Willimott and Moore [1926] and by Euler and his collaborators [1926]. The former observers ascribe the formation of two distinct colours to the varying proportions of phenol

[1] This fact agrees with and explains the recent observation of Moore and Willimott [1926], according to which Fearon's reaction persists in cod-liver oil after treatment with concentrated H_2SO_4, whilst the $AsCl_3$ reaction disappears together with vitamin A, which is destroyed by the acid.

used and even suggest that in one stage two "vitamin" molecules combine per phenol molecule, whilst in the other stage only one such molecule combines.

We are able to offer a much simpler explanation, since we found that these green and blue colours are not connected with the chromogen of Fearon's reaction at all, but are given by certain oils when mixed with a solution of pure trichloroacetic acid itself. The test, therefore, does not fulfil the essential condition, laid down by Fearon, that the condensing agent should not produce any colour with the oil in the absence of a phenol. When these dark green or blue solutions are shaken with a small volume of water, the blue component descends into the watery phase, whilst the yellow pigment (lipochrome?) remains in the light petroleum. Further, the unsaponifiable portion of all active liver oils gives with pure trichloroacetic acid (in concentrated aqueous or light petroleum solution) a blue colour, identical in all respects with the blue colour in the $AsCl_3$ or $SbCl_3$ reactions, described by Rosenheim and Drummond [1925] and by Carr and Price [1926].

It is evident, therefore, that Fearon's reagents react in many oils with two entirely different chromogens, and this fact alone makes it impossible to use as a colorimetric standard a buffered solution of phenol red, as suggested by Fearon, or the mixture of dyes proposed by Willimott and Moore.

We overcame this difficulty by employing Lovibond's colorimeter, in which the amount of red colour present in a solution together with blue and yellow can be accurately measured and expressed in units of red standard tints. There is no need to describe in detail the arbitrarily selected conditions, under which we were able to obtain comparative colour values for a large number of liver oils and fish body oils. It is sufficient to mention that they varied in intensity from 0 to 80 red units in a 10 mm. layer, for 100 mg. of oil, contained in 5 cc. solvent. The results of parallel feeding tests on rats showed that the colour values bore no relationship whatever to the vitamin content of the oils tested. Two extreme cases only need be mentioned. The fat of a pig's liver gave no colour reaction with Fearon's reagents, whilst its growth promoting value in the animal test was found to be twice that of a standard cod-liver oil. A sardine oil gave as intense a Fearon's reaction as the standard cod-liver oil, without producing growth or having any effect on xerophthalmia[1].

Summary.

1. Chemical and biological evidence is presented which shows that Fearon's reaction has no relation to vitamin A, for the estimation of which it was intended.

2. The suggestion is made that the chromogen of the reaction is associated with aldehydic oxidation products of unsaturated fatty acids, of the type of clupanodonic acid.

[1] We are informed by Prof. Drummond that he also found sardine oil devoid of vitamin A.

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CLXIX. ON THE NON-PROTEIN CYSTEINE IN PLANTS.

PRELIMINARY NOTE ON THE ATTEMPTED ISOLATION OF GLUTATHIONE FROM THE PEA (*PISUM SATIVUM*).

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(*Received October 7th, 1926.*)

Gola [1902] was the first to call attention to the colour reaction of some plant tissues with sodium nitroprusside and he suggested that it might be due to the presence of cysteine.

The present communication reports the attempted isolation of the substance responsible for this colour reaction.

Microchemical test. In order to find out which material was the richest in the substance giving the reaction, different plant tissues were compared in the following way. Small slices of tissues were ground with concentrated ammonium sulphate solution; a few drops of a freshly prepared 5 % solution of sodium nitroprusside were added and, after stirring, a drop of dilute ammonia. In many cases a fleeting purple-red colour developed immediately, and this was generally the case with the meristematic tissues of roots, with the ovules, and with young and ripe seeds. The substance responsible for the colour reaction could be extracted with hot water from the above material and was found in the filtrate after precipitation of the proteins.

In comparative tests it was found that the strongest colour reaction was given by the seeds of some *Leguminosae*, such as *Phaseolus multiflorus*, *Pisum sativum*, *Lupinus albus*, *Vicia faba* (not ripe), and also by the young seeds of many other plants, *e.g. Iris*, *Fritillaria*, *Scilla*, *Papaver*, *Acer*, *Melandrium*, *Primula*, *Helianthemum* sp. Much weaker reactions were obtained from the etiolated stems of asparagus, from potato tubers (*Solanum tuberosum*), from the roots of radish, from the latex of *Lactuca sativa* and from the green shells of the unripe fruit of peas and beans.

In some other cases the colour was different; for instance, that given by the scales of onions (*Allium Cepa*) was a violet, but if the juice which was squeezed out from them was mixed with one drop of nitroprusside solution it gave, after addition of ammonia, an intense golden yellow colour. When using the scales of garlic (*Allium sativum*) a bluish sediment appeared after some time. The fresh green leaves gave an olive green colour.

The seeds of the pea were used for the attempted isolation, described

below, of the substance responsible for the colour. An extract of the peas still gave, after precipitation of the proteins, the purple-red colour with nitroprusside and contained organic sulphur. Therefore it seemed probable that a simple compound containing an SH group such as cysteine or glutathione was present, and to decide which of these was actually present extraction and isolation based on Hopkins' method [1921] were undertaken.

Isolation of cysteine from the pea.

About 50 pounds of ripe green peas were soaked in tap-water for 24 hours and were then extracted three times with boiling water. After the hot extract had been strained through muslin, acetic acid was added and the precipitated proteins were filtered off. To the filtrate (50 l.) a concentrated solution of neutral lead acetate was added with constant stirring until no further precipitate was formed. The subsequent treatment of the lead precipitate was essentially that described by Hopkins for the isolation of glutathione. Believing, however, that part of the substance sought was present in the oxidised form, which (in the case of glutathione) is not thrown down by lead acetate, I precipitated the filtrate from the lead precipitate with mercuric sulphate. Both precipitates were decomposed with H_2S and the filtrate from the sulphide received in each case separate but identical treatment. After removing H_2S a concentrated solution of uranium acetate was added until excess was shown on testing with ferrocyanide solution. After filtering off the precipitate, a concentrated solution of $Ba(OH)_2$ was added; the precipitate thus formed was filtered off, and the baryta removed from the filtrate by means of H_2SO_4. The filtrate was precipitated with a 10 % solution of phosphotungstic acid (dissolved in 5 % sulphuric acid), and the filtrate was freed from the excess of phosphotungstic acid by means of a hot concentrated solution of $Ba(OH)_2$, and the excess of this was removed by H_2SO_4. The filtrate was now precipitated by acid mercuric sulphate, the precipitate, after being washed with dilute mercuric sulphate, was suspended in water and decomposed with H_2S. The mercuric sulphide was filtered off, and the filtrate freed from excess of H_2S by bubbling hydrogen through it. It was now precipitated with neutral lead acetate, and the precipitate decomposed with H_2S. The last two steps—precipitation by Hg and Pb—were repeated, and, finally, the fluid was neutralised with $Ba(OH)_2$ and air was blown through until the reaction with nitroprusside quite disappeared. The solution was exactly freed from Ba, and filtered into absolute alcohol. After 24 hours a white precipitate was separated by centrifuging, washed with alcohol and dried in a vacuum desiccator.

The yield was 28 mg. from the first lead precipitate and 200 mg. from the first mercury precipitate.

Properties of the isolated substance. The product which was obtained was a white, non-hygroscopic powder, easily soluble in water and insoluble in alcohol, ether and acetone. When dissolved in a small amount of water it

gave a yellow solution, which, after evaporation in a vacuum desiccator, left a yellow transparent amorphous mass behind and a small amount of a crystalline material which was mainly in the form of small sharp-pointed prisms gathered together in spheroids. After addition of water the crystals remained undissolved and were separated by filtration.

Hydrolysis. To the filtrate hydrochloric acid was added until it was present to the extent of 25 %. After being boiled for eight hours, the solution was cooled in ice and saturated with hydrochloric acid gas. It was placed in the ice-chest, but, as no crystals were deposited after 24 hours, the solution was concentrated under reduced pressure at 40–50° and again saturated with hydrochloric acid gas while being cooled on ice. The glistening white crystals were allowed to settle out for 24 hours in the ice-chest. They were separated by centrifuging, washed with cold concentrated HCl and also with cold alcohol saturated with HCl, and then dried over calcium chloride in a vacuum desiccator. Under the microscope the crystals appeared as long transparent flat parallelograms arranged singly and gathered together in rosettes. There were also present flat prisms unsymmetrically pointed at both ends. The substance melted at 204° (uncorr.) with decomposition, and at 213·5° (uncorr.) after repeated purification. When dissolved in a small amount of water, the crystals soon deposited an insoluble substance in the form of small needles gathered together in spheroids; after evaporation in a vacuum desiccator the residue appeared to be composed of spheroids and hexagonal symmetrical plates. When the substance was heated over a flame on a platinum foil it burned with a greenish flame without melting. It was insoluble in water, alcohol, acetone, ether and acetic acid; soluble in dilute ammonia and mineral acids. When heated in a capillary tube it blackened above 213° without melting. It was found to give the tests for a sulphur-containing compound and also a bluish violet colour with ninhydrin in neutral solution. When dissolved in dilute ammonia and acidified with acetic acid, addition of acetone caused a precipitate of small cubes, and when it was precipitated from its solution by means of mercuric sulphate and the precipitate suspended in water was decomposed with H_2S, a filtrate was obtained which reacted in the same manner with nitroprusside as cysteine.

These properties proved the substance to be cystine.

The mother liquors after the fractional separation of cystine hydrochloride left on evaporation under diminished pressure at 50° a brown amorphous substance which was insoluble in water.

Besides the cystine no other amino-acid could be identified in the products of hydrolysis of the substance isolated from peas, although from some hydrolyses of the original substance a small amount of crystals, resembling the hydrochloride of glutaminic acid, was obtained, melting at 203° without blackening. No further work could be carried out on them on account of the smallness of the yield.

Isolation of non-protein cysteine from grey "maple" peas. The ripe seeds

(25 kg.), without being previously soaked, were ground and extracted with boiling 1 % acetic acid. The extract was purified by the means described above.

The yield of substance was smaller than from the green peas and the only product of hydrolysis that could be identified was cystine.

Isolation of cystine from the leaves of "maple" peas. Although the leaves of the peas gave a negative nitroprusside reaction it was thought that this was not conclusive evidence that cystine was absent and this supposition was justified by the following experiment.

About 10 kg. of fresh green stems together with leaves (fruit and yellow leaves being separated), which were collected before the seeds were ripe, were extracted three times with boiling water. The extract was precipitated with acid mercuric sulphate, and the precipitate decomposed by H_2S. The filtrate from the sulphide was purified by the method above described. A very small amount of substance was finally obtained which gave the nitroprusside reaction and also the reaction for sulphur.

Discussion.

The above described work shows that non-protein cysteine is present in the seeds of peas. On the ground of microchemical examinations it seems likely that this substance is widely distributed in the plant kingdom. On account of the small yield of the substance, it was difficult to establish whether the cysteine and cystine respectively are present in the pea tissues in the free state or bound to some other compound. It is certain, however, that the cysteine which is responsible for the colour reaction with ammoniacal sodium nitroprusside is not in the form of a protein. Although the proteins which were precipitated from the extract by means of phosphotungstic acid, acetic and trichloroacetic acids still gave the colour reaction, the substance responsible for it could be gradually, although not completely, separated by redissolving and reprecipitating the proteins. It is possible that cysteine, or the compound in which it is present, was adsorbed by the protein. On the other hand, in addition to the cysteine, there may also exist in the pea and other plants a protein which, after denaturation, gives the nitroprusside reaction like denaturated egg-albumin [Harris, 1922] or philothion-β [de Rey Pailhade, 1926], but before this can be decided further work must be done.

It is very probable that the cysteine found in the pea is not in the free state but is bound with glutaminic acid, as in yeast and mammalian tissues.

Summary.

1. The pea (*Pisum sativum*) contains non-protein cysteine in the ovules, young and ripe seeds, and in the green shells of the unripe fruit; cystine is present in the leaves.

2. From histochemical studies it is very probable that cysteine is wide-

spread in the higher plants, not only in the meristem as Gola suggested, but also in the "reserve" tissues and in latex.

3. By the microchemical colour reaction of plant tissue with ammoniacal sodium nitroprusside it is not only possible to detect cysteine but also to follow histochemically its oxidation in the living tissues.

4. It is very probable that in the pea the cysteine is bound with glutaminic acid as a dipeptide such as glutathione.

5. The presence of free cysteine and of proteins corresponding to philothion in the pea is not excluded.

In conclusion, the author wishes to express his indebtedness to the Rockefeller Foundation for a fellowship, and also to Sir F. G. Hopkins for his kindness and the interest he has shown in this work.

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CLXX. A RAPID AND RELIABLE TEST FOR VITAMIN D.

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Zucker and Matzner [1923] have stated that rats kept on a typical high-calcium low-phosphorus rachitogenic diet develop marked faecal alkalinity, and that the administration of an antirachitic to rats in this condition causes the faeces again to become acid ($p_H < 7{\cdot}0$).

Although not specifically put forward as a means for determining antirachitic activity, its use for this purpose was suggested to us by the above authors, and after three years' experience we have found it to be of value when a more rapid test than those usually employed is required for routine purposes.

We have made use of the following technique.

Inbred albino rats from 20 to 40 days old and from 40 to 60 g. bodyweight are fed *ad lib.* on diet 401 [Zucker, 1924] which has the following composition:

Patent flour	85·0 %
Powdered egg albumin	10·0 %
Calcium lactate	2·8 %
Ferric citrate	0·2 %
Sodium chloride	2·0 %

with 2–3 g. fresh spinach leaves per animal per day.

It will be seen that this diet includes the addition of a reasonable amount of fat-soluble A. It has been shown by Chick and Roscoe [1926] that 2 g. of spinach, particularly in the winter, contain negligible amounts of vitamin D. We first used diet 84 [Sherman and Pappenheimer, 1921], but comparative experiments showed that the effects of the two diets were practically identical, while the animals on 401 seemed somewhat healthier and livelier than those on 84.

The cages used have a false bottom, consisting of a zinc screen with a mesh large enough readily to permit food to fall through, but small enough to retain the faeces. The faeces are collected before mid-day, any contaminated with urine being rejected; they are weighed, and placed in small stoppered cylinders with sufficient water to give a 2 % suspension. The faeces are well disintegrated with a glass rod, and vigorously shaken, and the suspension is then run through a loose plug of cotton wool, and poured into a hydrogen

electrode of the rocking pattern. The hydrogen ion concentration of the suspension is then determined electrometrically in the usual manner.

Determinations of this kind are made at three to four day intervals until the p_H of the faecal suspension is nearly or definitely alkaline (10 to 12 days). Determinations are then made every day, or every other day at least, until two consecutive readings give a mean determination of 7·3 or more, neither of the individual determinations being less than 7·2. The animal is now ready to be fed with the substance to be tested.

After the third day of administering the substance to be tested, readings are taken every day, until two consecutive readings give a mean value of p_H 6·7 or less, and neither more than p_H 6·8. For our own laboratory convenience we have considered the quantity of antirachitic substance necessary for this purpose to be an antirachitic unit.

1. *The rise in faecal p_H on the basal diet.* Since the test was first adopted by us, over 280 groups of 4 animals have been used. In no single case has the faecal p_H failed to reach a value well above 7·0. The time taken has been from 10 to 15 days, and the alkalinity has been maintained either till the end of the period of feeding the rachitogenic diet or until the animals have been submitted to antirachitic conditions.

2. *Maintenance of the alkaline p_H with supplementary doses of non-antirachitic substances.* The diagrams in Fig. 1 show the negative effects of administering glycerol, liquid paraffin, olive oil solutions of crude cholesterol from cod-liver oil, and an olive oil solution of recrystallised cholesterol (M.P. 148°).

3. *The restoration of acidic p_H by means of sources of antirachitic vitamin.* Fig. 2 shows the effect of administering cod-liver oil and irradiated cholesterol. The sample of oil in question had been found on four pairs of animals (2 males and 2 females) to give an average increase in calcification of 76 % over the controls, and to raise the average value of A/R [Chick and Roscoe, 1926] from 0·607 to 1·064.

4. *The restoration of an acidic p_H by means of irradiation.* Two pairs of animals, treated respectively for 15 and 30 minutes daily with the ultra-violet radiation from an open tungsten arc at 2 ft. distance, showed the change in faecal p_H illustrated in Fig. 3, curves 13 and 14.

5. *The prevention of the rise in faecal p_H by means of irradiation.* Two groups of two animals treated respectively for 15 and 30 minutes daily with the ultra-violet radiation from the tungsten arc at 2 ft. maintained their faecal acidity for 25 days, as shown in Fig. 3, curves 15 and 16.

We are aware that similar effects to those described above as a result of the administration of antirachitic substances have been obtained by the use of physiologically acidic substances, *e.g.* ammonium chloride [Zucker, Johnson and Barnett, 1922], or by considerably diminishing the calcium-phosphorus ratio of the diet [Sherman and Pappenheimer, 1921]. This does not, however, detract from the usefulness of the test in the case of substances of known origin, such as the extract of cod-liver oil to which we have been applying it for over two years.

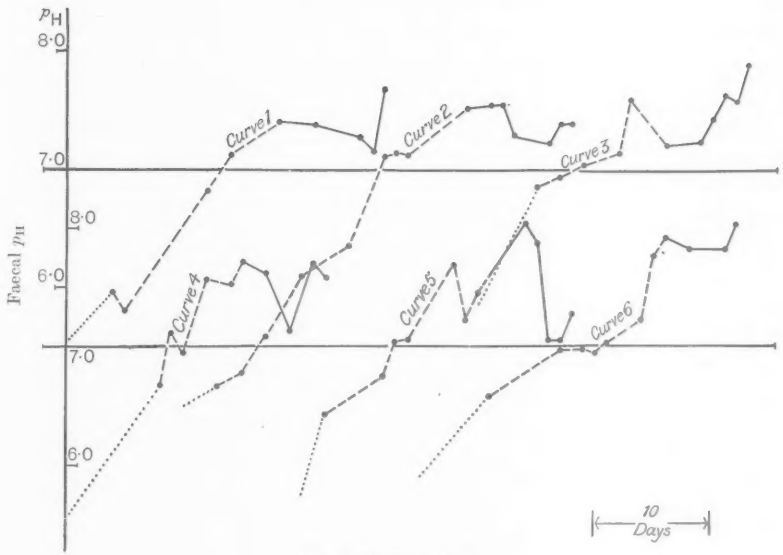

Fig. 1 (Curves 1 to 6).

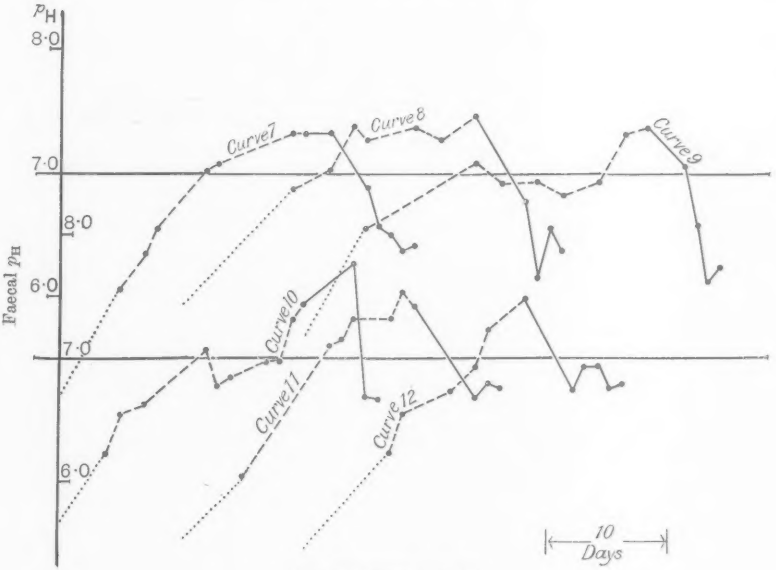

Fig. 2 (Curves 7 to 12).

– – – – – – – – = Period on basal diet alone.
———— = Period on supplemented basal diet.
. . . . . = Period prior to first determination.

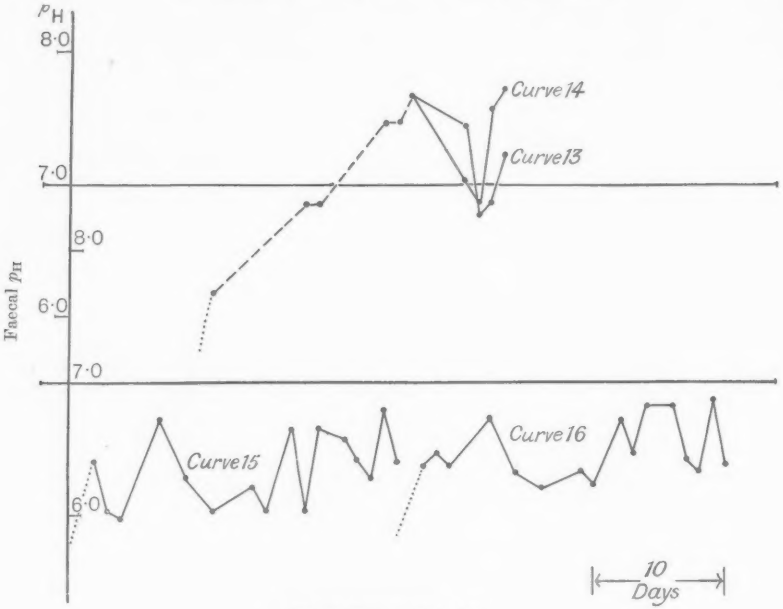

Fig. 3 (Curves 13 to 16).

---------- = Period on basal diet alone.
———————— = Period on supplemented basal diet.
..... = Period prior to first determination.

Daily supplementary treatment	Basal diet	No. of animals	Day of beginning supplementary treatment	Curve
Fig. 1				
0·04 cc. paraffinum liquidum, B.P.	401	2♀	18	1
0·025 cc. glycerol	84	2♂, 1♀	24	2
0·025 cc. glycerol	84	2♂	16	3
>0·001 g. recryst. cholesterol, in 0·02 cc. olive oil ...	401	2♀	15	4
>0·001 g. crude cholesterol, from cod-liver oil, in 0·02 cc. olive oil	401	2♀	15	5
>0·0015 g. crude cholesterol from cod-liver oil, in 0·02 cc. olive oil	401	2♀	22	6
Fig. 2.				
0·10 cc. cod-liver oil	401	1♂, 1♀	20	7
0·002 g. cholesterol, irradiated 1 hour, in 0·08 cc.	401	2♂	24	8
0·001 g. cholesterol, irradiated 2 hours, in 0·04 cc.	401	2♂	28	9
0·0005 g. cholesterol, irradiated 3 hours, in 0·02 cc.	401	2♀	20	10
0·00013 g. cholesterol, irradiated 4½ hours, in 0·08 cc.	401	2♂	19	11
0·002 g. cholesterol, irradiated 12 hours, in 0·08 cc.	401	2♀	18	12

All cholesterol was irradiated at 7½ inches from an open tungsten arc, and then dissolved in olive oil.

Fig. 3.				
Animals irradiated 30 minutes at 2 feet	401	2♀	16	13
Animals irradiated 15 minutes at 2 feet	401	2♀	16	14
Animals irradiated 30 minutes at 2 feet	401	1♂, 1♀	0	15
Animals irradiated 15 minutes at 2 feet	401	1♂, 1♀	0	16

The same arc was used for irradiating the animals as for irradiating the cholesterol. In the case of animals illustrated by curves 15 and 16, irradiation was omitted on the 6th and 13th days (Sundays).

SUMMARY.

1. Albino rats on certain high calcium-low phosphorus diets develop marked faecal alkalinity in 10 to 15 days.

2. This alkalinity is not affected by the administration of certain non-antirachitic substances.

3. The faecal p_H can be reduced to the acid side of neutrality by means of cod-liver oil, irradiated cholesterol or irradiation.

4. The rise in faecal p_H on this diet can be prevented by irradiation.

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CLXXI. THE SEPARATION OF FATTY ACIDS.

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Introduction.

In some preliminary experiments carried out by Professor H. A. D. Neville, the results of which had been communicated to the author, it had been noted that phenylhydrazine and hydroxylamine reacted, under suitable conditions, with the natural fats, giving crystalline products and that the yield of these products appeared to differ considerably with variation in the nature of the fat used. This gave rise to the idea that possibly one kind of glyceride, *e.g.* those containing saturated acids, formed solid crystalline products with the reagents mentioned above, whilst other glycerides either failed to react or gave products which were liquid or, where a solvent was used, soluble under the conditions of the experiment. The preliminary experiments had, however, been entirely qualitative and there was no information as to the degree of separation, if any, which could be obtained.

Most of the qualitative experiments had been carried out with hydrazine and phenylhydrazine and consequently the action of these reagents was first investigated. A considerable amount of work was done, but, not only did these reagents prove useless for the purpose in view, but the author was also anticipated in publication, Van Alphen [1925] publishing a complete account of these reactions.

It is only necessary, therefore, to give a very condensed account of the investigations on these lines before dealing more fully with the reaction of hydroxylamine.

Experimental.

Hydrazine. Falciola [1914, 1920] had investigated the action of this reagent on the fats and had come to the conclusion that in all cases hydrazides were formed but that the unsaturated fatty acids are reduced at the same time. Van Alphen, in the paper mentioned above, repeated this work under varying conditions and confirmed the conclusions of Falciola, while the author's experiments gave further proof of the fact. The experiments were discontinued, since the reaction was obviously useless for the purpose in view if, say, oleic acid gives rise to the same hydrazide as stearic acid. In addition, the products from different acids did not appear to differ sufficiently in properties to offer any hope of a basis of separation.

Phenylhydrazine. Many earlier workers, including Baidowsky and Slepaka [1903], have noted and used the reaction of this reagent on esters in general. The reaction is mostly a simple one, though the workers mentioned above noted the formation of benzamide and other nitrogenous by-products when esters of benzoic acid were used.

In ignorance of work being carried out by Van Alphen, the author investigated the reaction of phenylhydrazine on esters of acetic, butyric, palmitic, stearic and oleic acids, both simple esters and triglycerides being used. The calculated quantities of the reagents were heated together in an oil-bath to 150° for 1½ hours, a stream of hydrogen being passed through the tube to prevent oxidation. After cooling and standing for some time, a solid product separated out in all cases. These products, at first red in colour, could be purified by crystallisation from alcohol. The yields were not good even when a single triglyceride was used and, this being the case, the reaction was obviously useless as a method for the separation of different acids. It was definitely ascertained that, under the conditions of experiment, phenylhydrazine causes no reduction of the unsaturated to saturated acids. Thus the product obtained from triolein and phenylhydrazine gave on hydrolysis a fatty acid with the iodine value of pure oleic acid.

Hydroxylamine. The two previous reagents having proved useless for the purpose in view, attention was turned to hydroxylamine. The action of this reagent on the triglycerides of the fatty acids is to give hydroxamic acids which are remarkably stable [Morelli, 1908]. In the preparation of stearohydroxamic acid from tristearin by the action of hydroxylamine in the presence of sodium ethylate, Morelli found that the sodium salt of stearohydroxamic acid separated out in practically theoretical quantity from alcohol.

The method adopted in the following work is essentially the same as that used by Morelli, only slight alterations in procedure being found of advantage.

Stearohydroxamic acid, $C_{17}H_{35}CO.NH.OH$. Since this acid is fairly easily prepared and as its sodium salt is insoluble in alcohol, it was decided to prepare a sample of it for comparison purposes. Hydroxylamine hydrochloride, 1·8 g. in boiling methyl alcoholic solution, was added to 1·3 g. of sodium in absolute ethyl alcohol. To this solution 5 g. of tristearin were added and the whole thoroughly shaken for some time. After an hour the whole was warmed on a water-bath to dissolve any solid which might have formed. After allowing to stand for 24 hours, an abundant white precipitate of sodium stearohydroxamate was formed. This was filtered off, washed well with alcohol and then dried over sulphuric acid. The suspension of the dry salt in water was gradually raised to boiling-point when a turbid liquid was obtained, showing that slight hydrolysis had occurred. The solution was then acidified with *N* acetic acid with constant stirring, using Congo red as indicator. After standing for an hour the precipitate was filtered off and washed well with water. The solid was then roughly dried between filter papers and recrystallised from alcohol three times. It was finally washed with ether and dried, when the solid stearo-

hydroxamic acid was obtained in the form of white crystals which gave a violet colour with ferric chloride in alcohol; M.P. 104° (Morelli, 104°).

Palmitohydroxamic acid, $C_{15}H_{31}CO.NH.OH$. This acid was prepared, using exactly the same amounts of reagent as for stearohydroxamic acid. A white crystalline compound was obtained which, after recrystallising from alcohol three times had M.P. 100° (Morelli, 99°). It gave a violet colour with ferric chloride in alcohol. The yield of sodium palmitohydroxamate and of the palmitic acid obtained from this by hydrolysis was practically theoretical and this fact seemed to indicate that we might have a method of separating these acids from others occurring in the natural fats. It seemed probable that the hydroxamic acids from the unsaturated and lower fatty acids might either be much more soluble or might even be unstable.

Butyrohydroxamic acid, $C_3H_7.CO.NH.OH$. To test whether the lower fatty acids give hydroxamic acids under the conditions described above and, if so, to test their solubility, the preparation of butyrohydroxamic acid was attempted.

Using exactly the same procedure as for stearohydroxamic acid, it was found that no solid separated even after allowing to stand for a week. Either sodium butyrohydroxamate is completely soluble in alcohol or it is not formed under the given conditions. To test this point, the sodium chloride was filtered off and the filtrate evaporated to dryness on a water-bath. On adding acetic acid to the solution in water of the solid obtained by evaporation, a vigorous evolution of nitrogen occurred. It seems probable that sodium butyrohydroxamate was formed but is easily decomposed.

It may be concluded from the above that mixtures of higher and lower saturated fatty acids should be easy of separation by this method. A trial was made with a mixture of 4·229 g. tristearin (= 4·105 g. stearic acid) and 1·387 g. tributyrin. A solution of 1·8 g. of $NH_2OH.HCl$ in 25 cc. methyl alcohol was added while boiling to 1·3 g. sodium in 45 cc. ethyl alcohol. To this the above weights of triglycerides were added and the whole well shaken. After an hour the mixture was warmed on a water-bath to dissolve any solid which had separated out. It was then allowed to stand for 24 hours. The solid separating was filtered off and washed with 5 cc. alcohol. This solid was dried over sulphuric acid and finally hydrolysed by dilute mineral acid. The fatty acid so obtained was solid. It was extracted with ether and, when dried, weighed 4·035 g. The theoretical yield of stearic acid is 4·105 g. Thus a practically quantitative separation of stearic and butyric acids was effected.

Since butyric acid can be easily separated from stearic acid by the above method, it was decided to repeat the experiment with an acid of the same series intermediate between butyric and palmitic acids.

Caprylohydroxamic acid, $C_7H_{15}CO.NH.OH$. It was not possible to obtain the triglyceride of caprylic acid in a pure state, and the ethyl ester was used.

Adopting the usual procedure, it was found that no solid matter (sodium caprylohydroxamate) separated out on standing, and it was concluded that

sodium caprylohydroxamate is soluble in alcohol. It should, therefore, be possible to separate stearic and caprylic acids by this method. To test the point a mixture of 1·917 g. tristearin (1 mol.) and 1·111 g. ethyl caprylate (3 mols.) was added to the solution of NH_2OH and sodium ethylate prepared as before. After standing for 24 hours the solid was filtered off, dried over sulphuric acid and then hydrolysed by dilute mineral acid. The fatty acid so obtained was extracted with ether and dried.

This acid weighed 1·825 g. whereas the theoretical yield is 1·835 g. stearic acid. The filtrate obtained above was evaporated to dryness on a water-bath and then hydrolysed by dilute mineral acid, extracted with ether and the solution dried. On distillation, a yield of 0·9055 g. of caprylic acid was obtained. The theoretical yield is 0·930 g. The separation of caprylic and stearic acids appears then to be quantitative. The fact that the yield of caprylic acid is not quite quantitative is probably due to the fact that this acid is somewhat soluble in water and the ether extraction was not complete.

Laurohydroxamic acid, $C_{11}H_{23}CO.NH.OH$. Since a separation of caprylic and stearic acids was satisfactory, it was decided to repeat the experiment with an acid still higher in the same series. For this purpose lauric acid was chosen.

As with caprylic acid, it was impossible to obtain the triglyceride in a pure state so the ethyl ester was used. In the first case, an attempt was made to prepare laurohydroxamic acid using the same method as for stearohydroxamic acid. A white crystalline substance was obtained which after repeated recrystallisation from alcohol had M.P. 82·5°. It gave a violet colour with ferric chloride in alcohol, was soluble in warm alcohol, fairly soluble in ether and practically insoluble in dilute alcohol. Analysis established that the substance was pure laurohydroxamic acid which from an examination of the literature does not appear to have been prepared before. The yield, however, was low and it was obvious that a theoretical separation of stearic and lauric acids by this method was impossible. The approximate limit of application of this method for the separation of the higher from the lower saturated fatty acids is plain. It lies somewhere between the C_8 and the C_{12} acids.

Oleohydroxamic acid, $C_{17}H_{33}CO.NH.OH$. An investigation of the action of hydroxylamine on oleic acid was next undertaken in the hope that oleohydroxamic acid would differ sufficiently from the corresponding stearic acid compound to enable a separation to be effected.

A methyl alcohol solution of 1·8 g. $NH_2OH.HCl$ was added whilst boiling to 1·3 g. sodium in 45 cc. ethyl alcohol. To this solution 5 g. triolein were added and the whole was well shaken. After one hour's standing it was warmed on a water-bath. Even after allowing to stand for several days no separation of solid matter took place. This might have been due to the fact that sodium oleohydroxamate is soluble in alcohol or it might not be formed under the conditions used.

Repeating the experiment with 10 cc. methyl alcohol and 25 cc. ethyl

alcohol and the same weights of reagents and triolein, a small yield of sodium oleohydroxamate was obtained. The free hydroxamic acid is a white crystalline substance; M.P. 61° (Morelli, 61°). It gives a violet colour with ferric chloride in alcohol. On hydrolysis with dilute sulphuric acid, oleic acid was obtained which had a theoretical iodine value.

From the above it is seen that hydroxylamine has no effect on the double bond of oleic acid. The action of hydroxylamine on other unsaturated acids has been studied by several workers [Rostoski, 1875; Thiele and Pickard, 1899; Posmir, 1903; Harries and Haarman, 1904]. With esters of higher acids at ordinary temperatures the evidence points to the direct formation of the hydroxamic acids, no reaction occurring between the hydroxylamine molecule and the unsaturated linkage. Oleic acid would seem to be normal in this respect.

Separation of stearic and oleic acids. Since sodium oleohydroxamate is fairly soluble in alcohol, a method of separating oleic acid from the higher saturated fatty acids based upon the action of hydroxylamine on the fatty acids should be possible, providing enough solvent is present to keep the sodium oleohydroxamate in solution.

To test this point a mixture of tristearin and triolein was used. Equimolecular amounts of these two substances were acted upon by enough hydroxylamine to combine with both. After treatment as for stearohydroxamic acid a substance was obtained which gave a violet colour with ferric chloride in alcohol and had M.P. 95°. On recrystallising again the melting point rose to 101°. The substance was then hydrolysed by dilute mineral acid and the fatty acid so obtained extracted with ether and dried. This acid was solid; M.P. 68·5° (stearic acid, M.P. 69°). It gave no iodine value.

There remained to test whether a quantitative separation of oleic and stearic acids is possible by this method. An equimolecular mixture of tristearin and triolein was treated with enough hydroxylamine to combine with all the triglycerides present. To 1·8 g. $NH_2OH.HCl$ in 25 cc. methyl alcohol were added while boiling 1·3 g. sodium in 45 cc. ethyl alcohol. To this were added 1·522 g. triolein (= 1·456 g. oleic acid) and 1·532 g. tristearin (= 1·467 g. stearic acid). The whole was shaken thoroughly and after standing for an hour was warmed on a water-bath. After 24 hours' standing some solid matter had separated out. This was filtered off. The solid was dried over sulphuric acid and finally hydrolysed by dilute mineral acid when it gave a fatty acid which was extracted with ether and dried. The weight of this acid obtained from the solid precipitate was 1·461 g.: theory requires 1·467 g. stearic acid.

The filtrate obtained was evaporated to dryness on a water-bath. The oil so obtained was hydrolysed by dilute mineral acid, extracted with ether and dried.

Yield: 1·459 g. fatty acid: theory requires 1·456 g. oleic acid.

It would therefore appear that the method gives complete separation of stearic and oleic acids.

For a perfect separation it is obvious that the weights of triglycerides and reagents, and the volume of solvent must be within certain limits. It was decided to keep the weights of reagents and the volume of alcohol fixed at 1·8 g. of $NH_2OH.HCl$ in 25 cc. methyl alcohol and 1·3 g. sodium in 45 cc. ethyl alcohol. Repeating the experiment described above, but using greater amounts of triglycerides for the same volume of solvent and weights of reagent, a less perfect separation was obtained. In one experiment where 2·001 g. triolein (= 1·915 g. oleic acid) and 2·014 g. tristearin (= 1·928 g. stearic acid) were the amounts used, there were obtained 2·110 g. of solid acid, indicating that some sodium oleohydroxamate had separated with the stearic acid compound.

Separation of palmitic and oleic acids. 1·8 g. $NH_2OH.HCl$ in 25 cc. methyl alcohol were added while boiling to 1·3 g. sodium in 45 cc. ethyl alcohol. A mixture of 1·5656 g. triolein (= 1·498 g. oleic acid) and 1·4275 g. tripalmitin (= 1·360 g. palmitic acid) was added. After 24 hours' standing the solid formed was filtered off, dried over sulphuric acid and hydrolysed by dilute mineral acid. The fatty acid so formed was extracted with ether and when dried weighed 1·337 g. The filtrate from the above was evaporated to dryness on a water-bath, hydrolysed, etc., and gave fatty acid weighing 1·513 g. The separation of palmitic and oleic acids appears to be as complete as in the case of stearic and oleic acids.

With smaller amounts of triglycerides than those used above the separation was less perfect. A mixture of 0·9418 g. triolein and 0·8485 g. tripalmitin was tried, using the same weights of reagents and volume of solvent as in the previous experiment. The solid fatty acid obtained weighed only 0·602 g., whereas the theoretical yield would be 0·808 g. palmitic acid. Thus the solubility of sodium palmitohydroxamate becomes of importance when only small amounts of tripalmitin are used.

With weights of triglycerides greater than those used in the first experiment, the separation was again not perfect. In one experiment with the same weights of reagents and the same volume of solvent, a mixture of 2·7646 g. triolein and 2·5200 g. tripalmitin was tried. The solid formed after 24 hours' standing was treated as in previous experiments and gave 2·6654 g. solid fatty acid, whereas only 2·4020 g. palmitic acid are theoretically required.

From the above trials it is seen that a quantitative separation of stearic and oleic acids and of palmitic and oleic acids can be accomplished by the method described, provided that the weights of substances used are adjusted to the weights of reagents and volume of solvent used. This method is quite simple and the only precaution necessary is to use only absolutely pure and dry alcohol and to keep the apparatus used fitted with good calcium chloride tubes. After the sodium salt of the hydroxamic acid has once been prepared there is no further need to exclude moisture.

Variation in the proportion of glycerides. Both in the case of the stearic-oleic mixture and the palmitic-oleic mixture, experiments were tried with

varying proportions of saturated to unsaturated acid. The proportions used varied between three parts of saturated to one of unsaturated glyceride and one part of saturated to three parts of unsaturated glyceride. The separation was satisfactory in all cases when the total concentration of the fats in the mixed alcohol solvent was kept at about 4–5 %.

Application to natural oil. The method was tried with palm oil and castor oil, an indication of the extent of the separation being obtained from the iodine value of the products. A hot solution of 1·8 g. $NH_2OH.HCl$ in 25 cc. methyl alcohol was added to 1·3 g. sodium in 45 cc. ethyl alcohol. To this solution were added 4 g. palm oil. The reaction mixture was worked up as in previous cases. The iodine values of the original oil and of the two products were then determined. A similar experiment was carried out using 4 g. of castor oil. The results obtained may be given in tabular form:

	Iodine value of oil	Iodine value of acid from solid precipitate	Iodine value of acid from filtrate
Palm oil	19·53	Nil	26·53
Castor oil	83·39	Nil	100·03

It cannot be proved from the above that a perfect separation has occurred, as some saturated acids may have remained in solution, making the iodine value of the fatty acid obtained from the filtrate low. However, the separation is satisfactory so far as it gives saturated acids only in the precipitated hydroxamic acid.

Hydroxamic acids from hydroxy-fatty acids. As hydroxy-fatty acids quite commonly occur in nature, it was considered desirable to investigate them from the point of view of this reaction, *i.e.* to see if they gave sodium hydroxamates which were either soluble or insoluble in alcohol. It was decided to use a natural oil having a fairly high acetyl value for this purpose and rape oil was chosen. A sample of oil was first saponified by alcoholic potash and the free fatty acids obtained. After drying, the acetyl value of these fatty acids was determined and found to be 16·1.

The oil was then acted upon by hydroxylamine in the presence of sodium ethylate as in the experiments previously described, 5·3067 g. rape oil being used. The solid precipitate obtained after 24 hours' standing gave 0·5217 g. fatty acid on hydrolysis. This fatty acid had no acetyl value. The filtrate from the above, after working up in the usual way, gave fatty acids of acetyl value 18·3. It would appear therefore that the hydroxy-acids present in rape oil give hydroxamic acids whose sodium salts are soluble in alcohol and the above method can be used for separating hydroxy-fatty acids from acids which give hydroxamic acids having insoluble sodium salts.

Summary.

1. Phenylhydrazine reacts with the fatty acids and their triglycerides to give the corresponding phenylhydrazides. This applies to both saturated and unsaturated acids, the double bonds of the latter not being attacked, but the

products, owing to the great similarity of their solubility in various media, are unsuitable as a means of separating fatty acids.

2. Hydroxylamine, in the presence of sodium ethylate, reacts with the triglycerides of the fatty acids to give stable hydroxamic acids.

3. The sodium salts of the hydroxamic acids from palmitic and stearic acids are completely insoluble in alcohol under the conditions stated but, with decrease of molecular weight, the solubility increases, the corresponding compound from lauric acid being appreciably soluble whilst that from caprylic acid is totally soluble.

4. The sodium salts of the hydroxamic acids from the unsaturated fatty acids and from the hydroxy-fatty acids are completely soluble under the given conditions.

5. The reaction, therefore, offers a method of separation of saturated fatty acids of high molecular weight, certainly from palmitic acid upwards from (*a*) the lower acids such as butyric and caprylic, (*b*) the hydroxy-acids, and (*c*) the unsaturated acids, in cases where all these may occur in natural oils in the form of glycerides.

6. For the separation to be satisfactory, attention must be paid to the concentration of the reagents, a concentration of about 4–5 % in the mixed alcohols being the best. In addition, the methyl and ethyl alcohol used, particularly the former, must be pure and dry.

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